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COMPLEMENT FIXATION IN ANIMAL NEOPLASIA. II. DEVELOPMENT AND DURATION OF THE REACTION IN NEW ZEALAND WHITE RABBITS CARRYING BROWN-PEARCE CARCINOMA.[1 2 3]

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In a previous paper (*1*) we reported that by modifying the complement-fixation test described by Kidd (*2*) many positive reactions were obtained when the sera of New Zealand White rabbits bearing the Brown-Pearce carcinoma were tested with saline extracts of the neoplasm as antigens. Kidd obtained 80 percent positive reactions with "mostly 'blue-cross' and agouti hybrids" bled approximately 31 days after inoculation. However, later work (*3*) indicated that few market-bought breeds of rabbits developed the strongly positive reactions obtained with "blue-cross" hybrids. These findings of Kidd and his co-workers with market-bought rabbits (of which the New Zealand White breed is an example) were borne out by the results we obtained when New Zealand White rabbits carrying the neoplasm were tested by following the technique of Kidd as closely as possible; only very few weakly positive reactions were obtained. With the more sensitive and precise modification, however, the percentage of positive reactions with market-bought New Zealand White rabbits was of the same

[1] Received for publication August 13, 1951.

[2] Presented in part at the 40th annual meeting of the American Association for Cancer Research, Detroit, Mich., April 16 and 17, 1949.

[3] Presented in part at the Gordon Research Conference on Cancer, New London, N. H., August 28-September 1, 1950.

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order of magnitude as that reported by Kidd when he used "blue-cross" and agouti hybrids. In this paper we are reporting on the development, duration, and specificity of the reaction with sera of New Zealand White rabbits.

METHODS

Heavy cell suspensions of the neoplasm were prepared as previously described (*1*), and adult male animals were inoculated in multiple sites with 0.5-ml. portions of this suspension. Palpable tumors appeared in nearly every case, usually by 1½ weeks. Before inoculation with the neoplasm, 20-ml. specimens of blood were obtained by cardiac puncture in order to provide preinoculation (normal) sera; after inoculation similar bleedings were made at weekly intervals to provide postinoculation (tumor) sera. Of the 190 animals examined serologically, 33 percent died within 4 weeks after inoculation and another 14 percent were sacrificed during this period. Thirty-six percent of the animals died or were sacrificed within 5 to 7 weeks after inoculation. Seventeen percent of the animals died or were sacrificed within 8 to 20 weeks after inoculation. Postmortem examinations were made on 95 percent of these 190 animals, and sections for microscopic examination were taken from 85 percent of the animals. In 97 percent of the animals living 2 to 7 weeks after inoculation grossly visible tumors were present; microscopic examination of these neoplasms showed the typical histologic features described by Brown and Pearce (*4*). Although palpable tumors had been observed during life in most of the animals surviving longer than 7 weeks, grossly visible tumors were found at autopsy in only 16 percent of these animals; apparently the neoplasms had regressed in the remaining 84 percent. Histologic evidence (degeneration, histiocytic reaction, and calcification) of such regression was noted in several of the microscopic sections taken from the inoculation sites.

The complement-fixation test employed consisted of a series of complement titrations, both with and without antigen, in the presence of the test serum and in the presence of a reference pool of normal rabbit serum. The difference between the increase in complement requirement of the test serum due to the presence of antigen and the increased complement requirement of normal serum due to the presence of antigen was expressed as a titer. The tests were performed under the conditions that had been shown previously (*1*) to be most satisfactory.

The fresh or frozen sera were inactivated at 61° C. for 30 minutes before being diluted, the tests were incubated at 5° C. for 18 hours to allow fixation to occur, 2-percent sheep cells sensitized with the optimum hemolysin concentration as determined under the conditions of the test were added, and the degrees of hemolysis were determined after a 30-minute incubation period at 37° C. The titer of each serum specimen has been calculated by subtracting from the amount of complement (ml. of a 1:100 dilution) required for 50-percent hemolysis in the presence of test serum and antigen the sum of the following: (1) The amount required with test serum alone, and (2) the amount required due to the anticomplementary effect of antigen in the presence of the reference pool of normal serum. The titer has been expressed as the number of units of complement "fixed" per ml. of test serum—actually 0.4-ml. amounts

of diluted serum were used in the test. The method used for calculating the titer is expressed in the formula $\frac{TS+A+[TS+(NRS+A-NRS)]}{IC}$. The symbols in the formula represent the ml. of 1:100 complement required in the following titrations:

$TS+A$ = test serum plus antigen.
TS = test serum alone.
$NRS+A$ = reference pool of normal rabbit serum plus antigen.
NRS = reference pool of normal rabbit serum (100 animals) alone.
IC = the immediate complement titration required to determine the size of the unit.

Because of small differences among individual normal sera, they may give titers of slightly more or slightly less than the zero titer of the reference pool. Test results were considered to be abnormal or positive when the titers obtained exceeded the highest expected normal titer (the mean of individual normal titers + 3 standard deviations) for the particular antigen preparation and serum-antigen ratio used.

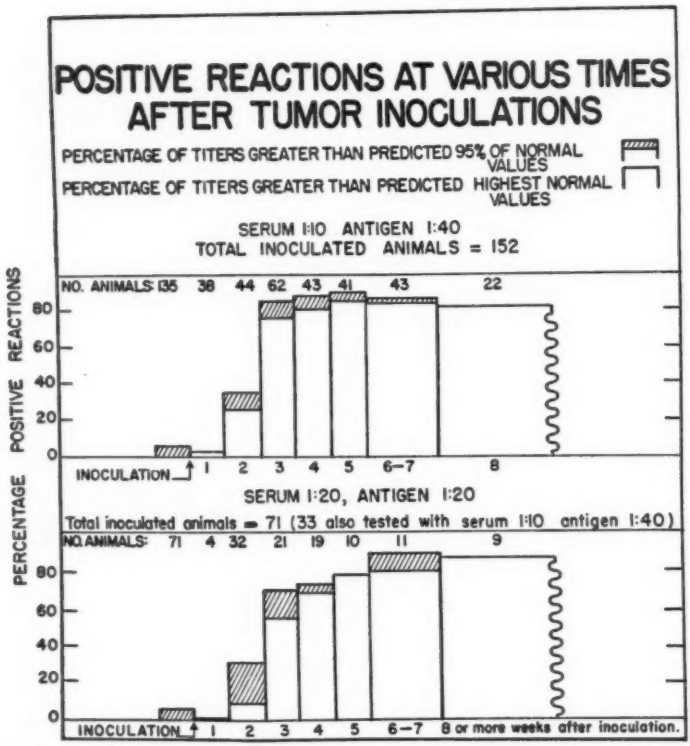
RESULTS

Positive Reactions at Various Times After Tumor Inoculation

The percentages of positive reactions found in routine tests of sera obtained from 190 animals at various intervals after inoculation of the neoplasm are shown in text-figure 1. Also shown are the percentages of titers that were in the "high normal" range (exceeding 95 percent of expected normal values). The number of animals from which the percentages were calculated are shown above each percentage bar.

The sera of 80 percent of the animals were tested by using a 1:10 dilution of the serum and a 1:40 extract of the tissue as an antigen—concentrations in the same range as those used by Kidd. In addition, the sera of 20 percent of the animals were tested at a 1:20 dilution with antigen diluted 1:20, while the sera of 17 percent of the animals were tested at both serum-antigen ratios. At the 1:10–1:40 ratio a few positive tests were obtained in the first 2 weeks, the percentage of positive tests increased sharply during the third week, and it reached a maximum of 86 percent 5 weeks after the inoculation of the neoplasm.

The 1:20–1:20 ratio appeared to be satisfactory for the detection of the more reactive sera obtained more than 4 weeks after inoculation, but the results shown in the figure indicate that tests performed at this ratio failed to detect some of the weakly reactive sera obtained within the first few weeks after inoculation—an abnormally large percentage of these sera gave titers that were only in the highest 5-percent-of-normal range. This conclusion is justified by the fact that several of the sera tested at both ratios gave positive tests at the 1:10–1:40 ratio and negative tests at the 1:20–1:20 ratio. The higher percentages of positive tests obtained with more concentrated serum (the 1:10–1:40 ratio) are in agreement with the results of other experiments which indicated that while the 1:10–1:40 ratio was adequate to detect most weakly reactive sera, still more would have been detected had the sera been tested at a 1:5 or a 1:2 dilution.


POSITIVE REACTIONS AT VARIOUS TIMES AFTER TUMOR INOCULATIONS
PERCENTAGE OF TITERS GREATER THAN PREDICTED 95% OF NORMAL VALUES
PERCENTAGE OF TITERS GREATER THAN PREDICTED HIGHEST NORMAL VALUES
SERUM 1:10 ANTIGEN 1:40
TOTAL INOCULATED ANIMALS = 152
NO. ANIMALS: 135 38 44 62 43 41 43 22
PERCENTAGE POSITIVE REACTIONS
80
60
40
20
0
INOCULATION
1 2 3 4 5 6–7 8
SERUM 1:20, ANTIGEN 1:20
Total inoculated animals = 71 (33 also tested with serum 1:10 antigen 1:40)
NO. ANIMALS: 71 4 32 21 19 10 11 9
80
60
40
20
0
INOCULATION
1 2 3 4 5 6–7 8 or more weeks after inoculation.

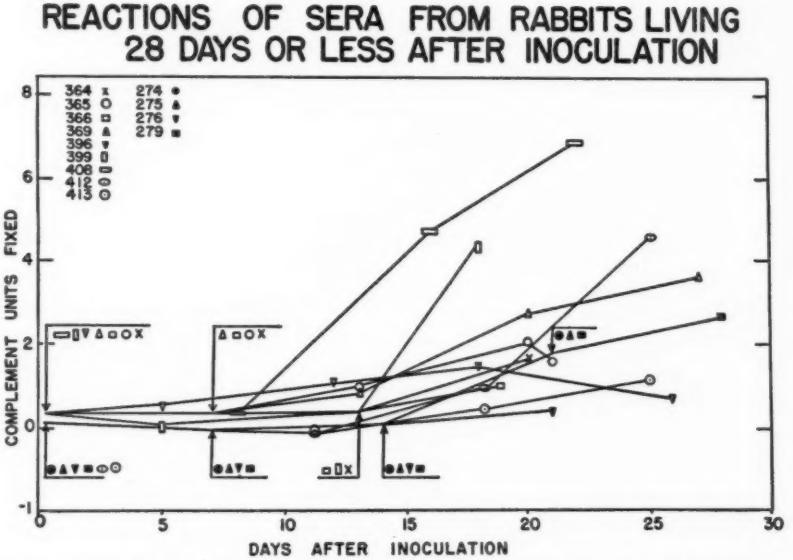
TEXT-FIGURE 1.—Percentages of positive tests obtained with individual serum specimens collected preinoculation and at weekly intervals postinoculation. Serum-antigen ratios: 1:10–1:40 and 1:20–1:20.

It seemed possible that the time at which positive reactions appeared and the extent to which they increased might differ between the group of animals dying relatively early with massive neoplastic growth and the group surviving for a considerably longer period of time with evidence of regression of the neoplasm. Therefore, the test results of sera obtained from the serial bleedings of animals in each of these two groups were compared by plotting their individual reaction curves. Text-figures 2 and 3 illustrate the extent to which the reaction developed in animals that lived 3 to 4 weeks, and in those that lived 10 or more weeks after inoculation. The tests on all animals in the first group were performed at the serum-antigen ratio of 1:10–1:40, but the limited amounts of some of the serum specimens obtained from four of the animals that lived 10 or more weeks necessitated the testing of all specimens from these animals at the 1:20–1:20 ratio. Since the reactivities of the sera covered a wide range and a constant serum-antigen ratio was used in tests of all sera from each animal, all specimens were not tested at the ratios that would give maximum reactions. Presumably, the titers obtained at the 1:10–1:40 ratio would be more reliable for weak reactions, and those at 1:20–1:20 would

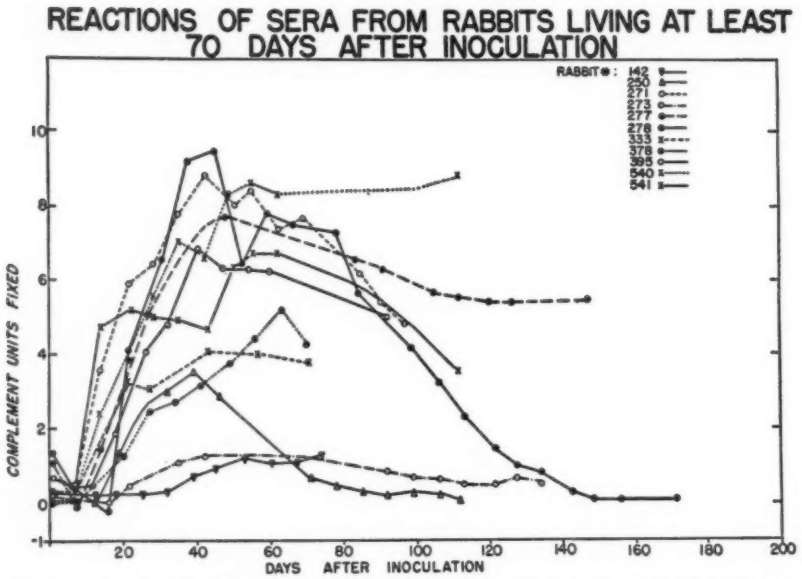
be more reliable for strong reactions. In order to plot on the same scale the development of the reaction in all of these animals, the results of the tests have been expressed as units of complement fixed at the serum dilution used, rather than as titers or units fixed per milliliter of undiluted serum.

In text-figure 2 are illustrated the reactions in all of the 13 animals that died within 4 weeks and from which at least 3 postinoculation serum specimens were obtained. In each case the complement requirements increased from the preinoculation value. In 2 cases (rabbits 276 and 413) the calculated titers did not increase enough to exceed the normal range for the antigen used, but at 21 and 25 days, respectively, they had reached the "high normal" range. One other animal (396) developed a positive reaction at 18 days postinoculation, but the titer calculated for the serum specimen obtained at 26 days was within the "high normal" range. The remaining 10 animals all showed positive reactions at the time the last blood samples were taken. In general, these reactions were of the same order of magnitude as those obtained at corresponding postinoculation times in the animals that survived 10 or more weeks.

In text-figure 3 are shown the reactions of sera from the 11 rabbits living 10 or more weeks on which there were results covering the complete period from inoculation to death. In each case the complement requirements



TEXT-FIGURE 2.—Development of the reaction in individual rabbits that died within 4 weeks after inoculation of the neoplasm. Serum-antigen ratio: 1:10–1:40. Open symbols—all serum specimens from any one animal tested on the same day. Sera from seven animals tested with antigen BPVII. Sera from animals 412 and 413 tested with antigen BPIX. Solid symbols—all serum specimens from any one animal not tested on the same day. These sera tested with antigen BPI.



TEXT-FIGURE 3.—Development and duration of the reaction in individual rabbits that survived 10 weeks after inoculation of the neoplasm. All serum specimens from any one animal tested on the same day. Animals 271, 273, 277, and 278 tested at serum-antigen ratio 1:20–1:20 with antigen BPIIIa; remainder of animals tested at serum-antigen ratio 1:10–1:40. Sera from animals 378, 395, and 333 tested with antigen BPVII; from animals 142 and 250, with BPIX; from animals 540 and 541, with antigen BPXVII.

increased from the preinoculation value. In one case (rabbit 273) the calculated titer never reached the "high normal" range, while in another case (rabbit 142) the titer did increase to the "high normal" range. The remaining 9 animals developed positive reactions, reaching their peaks at about 40 to 60 days; with the exception of 2 of these animals, the reactions then definitely decreased, and 2 animals gave negative reactions 3 to 4 months after inoculation.

From the data shown in these 2 figures it is evident that the reactions in the animals that died with massive neoplastic growth within 4 weeks after inoculation were approximately as great as those observed at the corresponding postinoculation period in the animals that survived for as long as 5 months. When the reactions were expressed in terms of titer, the highest 1:10–1:40 value (units fixed $\times$ 25) obtained with the animals in the 4-week group was 173. The highest 1:10–1:40 titer obtained with the animals in the long-term group was 220; the highest 1:20–1:20 titer (units fixed $\times$ 50), obtained with a different antigen, was 475. Eleven of the thirteen 4-week animals gave positive tests by 20 or 21 days; 8 of the 11 long-term animals also gave positive tests by the same time. Two of the 4-week animals failed to develop positive reactions, although extensive

neoplastic growth was found at autopsy. Two of the long-term animals also failed to develop positive reactions; 1 of these animals had palpable tumor 1 month after inoculation, although no characteristic neoplastic cells were found at autopsy. The other long-term animal was not examined during the postinoculation period, and only the inoculation sites were found *post mortem*.

SPECIFICITY OF THE TEST

The antigens used in tests reported in this paper were saline extracts of pools of neoplastic tissue obtained from about 20 rabbits. As has been noted, the postinoculation sera of most rabbits gave positive reactions with these pooled antigens. To answer the question as to whether or not the serum of an individual rabbit gives a positive reaction with a saline antigen prepared from its own neoplasm, postinoculation sera from 11 rabbits were tested simultaneously with antigens prepared from their corresponding neoplasms and with antigen pool BPIX. Nine of the 11 sera gave definitely positive reactions at a 1:10 dilution, both with antigens prepared from their own neoplasms and with antigen BPIX. One serum gave a minus titer, and one gave a very low titer when tested with the corresponding antigen, although both gave definitely positive reactions with antigen BPIX. Doubling the serum concentration still gave a minus titer for the 1 serum and a very low titer for the other. The low titers obtained with these 2 sera were not due to inactivity of the antigens, for all of the individual antigens gave definite reactions with a pooled tumor serum (TSXI). It appears that a postinoculation serum that gives a positive complement-fixation reaction with a pooled antigen will usually also give a positive reaction with an antigen prepared from neoplastic tissue obtained from the same animal.

The specificity of the usual reaction utilizing antigen prepared from pooled neoplastic tissue was investigated in 4 ways: 1) by testing the serum specimens obtained from 6 uninoculated normal animals that were bled serially in the same manner as were the animals carrying the neoplasm; 2) by testing serial specimens obtained from 14 animals inoculated with autoclaved neoplasm, with a necrotizing agent, or with normal rabbit tissues; 3) by testing single serum specimens from 53 animals inoculated with various infectious agents and/or foreign proteins; and 4) by testing serial specimens from 5 animals inoculated with cell-free filtrates of the neoplastic tissue.

Serial bleeding of normal animals.—Since 15–20 ml. of blood were removed from the tumor-carrying animals each week, it was important to determine whether or not simply the weekly removal of this amount of blood would alter the serum so that positive reactions would be obtained. As a matter of fact, by the eighth bleeding of the normal animals there were small differences of about 10 percent in the erythrocyte and leukocyte counts, and in the hematocrit, hemoglobin, and serum protein values. Complement-fixation tests were performed on serum specimens obtained weekly for a period of 8 weeks from each of 6 normal rabbits. There were

no positive reactions. All the titers were within the normal range for the antigen used.

Serial bleeding of inoculated animals.—The possibility exists that positive reactions might be obtained following the multiple inoculation of rabbit tissues other than the viable neoplasm, or that necrosis and inflamation alone would give rise to positive reactions. Therefore, multiple inoculations of each of the following substances were made into each of two rabbits: Turpentine, autoclaved Brown-Pearce carcinoma, and normal rabbit kidney, liver, spleen, testicle, or muscle. The sera were first tested at a serum-antigen ratio of 1:20–1:20 and, when the amount of serum permitted, the test was repeated at the more sensitive ratio of 1:10–1:40. No positive reactions were obtained at either of the usual serum-antigen ratios. All but one of the serum specimens obtained from one of the animals inoculated with rabbit kidney gave titers falling within the "high normal" range when tested at the 1:20–1:20 ratio. Two of these specimens were also tested at the usually more sensitive 1:10–1:40 ratio; the serum obtained 3 weeks postinoculation gave a "high normal" titer at both ratios while that obtained 5 weeks postinoculation was well within the normal range at both ratios. Even at the 1:20–1:20 ratio the course of titer development in this animal showed no resemblance to that observed following inoculation with viable neoplastic tissue, for the highest (still normal) titer was obtained 1 week after inoculation.

Single serum specimens from animals inoculated with various agents.—Positive reactions might be obtained with the sera of animals that had antibodies to specific antigens or of animals that had been inoculated with pathogenic agents. Therefore 53 single serum specimens from rabbits inoculated with a variety of agents were tested at the 1:10–1:40 ratio. The materials inoculated and the number of specimens tested were: *S. typhosa* (1 serum specimen), *Str. pyogenes* (9 specimens), *T. cuniculi* (8), *E. coli* (1), *S. aureus* (1), *Str. pyogenes* in B. C. G. adjuvant (1), *M. tuberculosis* var. hominis (3), B. C. G. in adjuvant (1), *Histoplasma capsulatum* (2), *Endamoeba histolytica* (2), *Schistosoma japonicum* (2), toxoplasma (1), Shope papilloma virus (1), vaccinia (3), herpes (2), Japanese B. encephalitis (1), M. M. encephalomyocarditis (2), poliomyelitis, SK-murine strain (1), psittacosis-lymphogranuloma venereum group (3), "fixed" rabies (1), streptolysin (3), human serum (1), horse serum (2), and bovine albumin (1).[4] No positive tests were obtained. The titer with the serum of 1 animal inoculated with *T. cuniculi* and the titer with 1 anti-M. M. (encephalomyocarditis group) virus serum were within the "high normal" range for the antigens used. These 2 sera were among the most anticomplementary of those tested.

[4] We are indebted to the following people for their kindness in supplying us with rabbit antisera included in this specificity study: Dr. R. S. Weiser, University of Washington, Seattle, Wash.; Dr. C. A. Evans, University of Washington, Seattle, Wash.; Dr. E. H. Lennette, Viral and Rickettsial Disease Laboratory, California Department of Public Health, Berkeley, Calif.; Dr. T. P. Hughes, Public Health Service, Communicable Disease Center, Montgomery, Ala.; Dr. A. N. Gorelick, Biological Department, Chemical Corps, Camp Detrick, Md.; Dr. H. A. Dettwiler, Eli Lilly Company, Indianapolis, Ind.

Inoculaion of cell-free filtrates.—Rivers and Pearce (*5*) reported the presence of Virus III in their stock Brown-Pearce carcinoma; it accompanied the neoplastic cells when they were transplanted to new hosts and multiplied there for at least 54 days. Therefore, two separate experiments were performed in this laboratory to investigate the possibility that the positive complement-fixation reaction with saline extracts of the neoplasm might be due to the presence of a "passenger virus" in our stock Brown-Pearce carcinoma. Control animals were inoculated in the usual manner with a neoplastic cell suspension, while the test animals received corresponding amounts of a filtrate of the same suspension obtained by passing a portion of it through a sintered glass bacteriologic filter, UF porosity.

In the first experiment the cell suspension used was inoculated into 2 control rabbits while 3 test animals received a filtrate of that suspension; the animals were bled at 9 weekly intervals and again when they were sacrificed at 16 weeks. In the second experiment a suspension of neoplastic cells from a different animal was inoculated into 2 control animals while 2 test animals received the cell-free filtrate; these animals were bled at 12 weekly intervals. In both experiments the control animals had palpable tumors during life, but by the time they were sacrificed the tumors had disappeared, leaving at the inoculation sites either a few recognizable neoplastic cells or the histologic evidences of regression already mentioned. None of the test animals showed palpable tumors at any time postinoculation, and sections of the organs and inoculation sites showed neither neoplastic cells nor the evidences of regression seen in the control animals. In each experiment 1 test animal died with hemopericardium at about 6 weeks postinoculation, before the experiment was completed. Both of the control animals (bearing neoplasm) in each experiment gave positive complement-fixation reactions from about 3 weeks postinoculation through the end of the experiment. None of the serum specimens from any of the 5 test animals (receiving filtrate) gave positive complement-fixation tests. If agents that would pass a sintered glass UF filter were present in the inoculated filtrates, their presence did not result in the development of the positive complement-fixation reactions that were observed in animals in which the neoplasm grew.

SUMMARY AND CONCLUSIONS

The sera of 190 New Zealand White rabbits bearing the Brown-Pearce carcinoma have been studied by the complement-fixation test previously described. The sera of 199 normal control animals and of 78 specificity control animals were also tested. A test was considered to be positive when the amount of complement required was greater than that which would be required by any normal serum examined with the same saline antigen at the same serum-antigen ratio. The serum-antigen ratios routinely used were not necessarily those which made the test most sensitive.

Positive reactions began to appear about 2 weeks after heavy inoculation with neoplastic tissue, shortly after palpable tumors had been noted. One-third of the animals died with massive neoplastic growth within 4 weeks, while about one-fifth survived for more than 7 weeks, usually with little or no distinguishable neoplastic tissue remaining. At 2, 3, 4, 5, and 6 weeks the percentages of positive tests were 18, 73, 82, 86, and 84, respectively. Of animals surviving for more than 7 weeks 83 percent gave a positive test on at least one occasion after this interval, but the neoplasm tended to regress in individual animals and the actual titers tended to diminish.

A postinoculation serum giving a positive complement-fixation reaction with the usual saline antigen prepared from pooled neoplastic tissue usually also gave a positive reaction with an antigen prepared from the rabbit's own neoplasm.

No positive reactions were given with the usual saline antigen by serial serum specimens obtained from 6 normal rabbits or from 19 animals receiving multiple inoculations of turpentine, of various normal rabbit tissues, of autoclaved Brown-Pearce carcinoma, or of cell-free filtrates from viable neoplastic tissue. The saline antigen did not give positive tests with 53 single serum specimens obtained from animals inoculated with various pathogenic agents or immunized against various foreign proteins.

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STUDIES ON AN ASCITES (RETICULO-ENDOTHELIAL CELL?) SARCOMA OF THE RAT [1] [2] [3] [4]

TOMIZO YOSHIDA, *Department of Pathology, Tohoku University Medical School, Sendai, Japan*

ORIGIN OF THE YOSHIDA SARCOMA

The Yoshida sarcoma originated in 1943 at Nagasaki in one of 20 rats treated as follows (*8*):

1) *o*-Amidoazotoluol (4′-amino-2,3′-dimethyl-azobenzene) feeding for 3 months.

2) Cessation of the feeding was followed immediately by painting with arsenite of potassium solution the skin of the back of animals three times a week. The solution consisted of equal amounts of absolute alcohol and Fowler's solution.

Toward the end of the third month of arsenite painting, a tumor was found growing in the scrotum of the rat. About a month later, the tumor became so large that it seemed to be on the verge of ulceration. The animal was sacrified for autopsy, and a malignant growth showing a widely spreading invasion through the pelvic cavity to the retroperitoneal tissues was found.

In the peritoneal cavity there was found a thick, milky ascites, which was actually a suspension of tumor cells. The ascites was transmitted into the peritoneal cavity of two other rats. One of them died the next day. The tumor grew in the remaining rat, giving rise to tumor ascites in the peritoneal cavity, and the picture of infiltration of cells into the surrounding tissues was similar to that of the original tumor. Successive

[1] Received for publication September 24, 1951.

[2] At the annual meeting of the Japanese Cancer Research Association in April 1948 it was decided that this tumor found by me in 1943 in Nagasaki, would hereafter be designated as "Yoshida sarcoma" to preclude confusion with other possible tumors.

[3] EDITOR'S NOTE.—A portion of this paper was given by invitation as part of the Gordon Cancer Conference of the Gordon Research Conferences on 30 August 1951, held at Colby Junior College, New London, New Hampshire, U. S. A.

[4] This paper includes an Addendum entitled: "Recent Trends of Cancer Research in Japan" by Waro Nakahara. Dr. Murray Shear, the Chairman of the Gordon Cancer Conference, made a suggestion in his letter that it would be of some value for the conference members, if I would additionally talk about the recent trends of cancer research in Japan. I asked Dr. Waro Nakahara, the President of the Japanese Cancer Association, to prepare for me a short note on this subject, because I was sure that he could do this task in much more excellent manner than I. He was kind enough to comply with my request. His note is presented as an Addendum to this paper.

transplantation with the tumor-ascites is being continued up to now, amounting to over 300 generations.

The massive tumor tissue of the original rat, which presented microscopically the picture of common round-cell sarcoma, was also transplanted into the subcutaneous tissue of normal rats. Localized massive tumor tissue developed at the site of grafting, as in the case of usual tumor transplantation, and successive subcutaneous transplantation with this material was continued throughout several generations. But, when a small piece of this subcutaneous material was introduced into the abdominal cavity of the animal, it always gave rise to the milky fluid growth, as in the ascites transplantation. Inversely, a droplet of the ascites introduced into the subcutaneous tissue caused a localized massive tumor at the site of inoculation.

The gross appearance of this tumor, either solid or fluid, depends upon the condition of the site of inoculation. Neither the solid nor the fluid characteristics of this tumor can be regarded as peculiarly essential to this tumor, each being only transient characteristics. At present, therefore, we maintain only the ascites form of the tumor in the laboratory because of the technical simplicity of successive transplantation and of the extensive availability of the ascites form for various purposes of investigations on cancer.

It was indeed good fortune that we have found such a peculiar tumor. However, I regard it as a by-product of our research on the "ascites-tumor," based on my working hypothesis of a "fluid tumor," which opinion I have stated in an article published in 1949 in Gann, the Japanese journal of cancer (*7*).

I may touch upon my own opinion concerning the manner of origin of this ascites tumor, termed for convenience the "Yoshida sarcoma." From the outset, I have been of the opinion that it was not a spontaneous tumor. The only fact giving sufficient basis for this belief is that no one has ever found such a tumor, despite the tremendous number of albino rats which are and have been examined by investigators all over the world. The same fact also provides reason for rejecting the suggestion of a virus origin of this tumor. We are still trying to reproduce the tumor.

The tumor was originated in 1943, as above described, in only 1 of 20 animals treated in the same way. The treatment was started by Muta at my suggestion in order to examine a possible combined effect of amino-azo dye and arsenite compound in the production of hepatoma. Since then, we have repeated many times the same manner of treatment so as to be able to reproduce the Yoshida sarcoma, and to study the manner of its origin—but always in vain.

Possible Relation of Yoshida Sarcoma to Tumors Derived from Kupffer's Cells

Last year, we obtained, at long last, another example of the tumor. This tumor originated, again by chance, in one of many rats which Tanaka (*4*) fed at my suggestion *o*-amidoazotoluol for about 300 days with the purpose of studying changes in the chromosomes of the liver cells in the

course of the development of hepatoma. The new tumor resembles in all respects the Yoshida sarcoma.

In view of this finding, it has become clear that the origin of Yoshida sarcoma is closely related to the development of aminoazo-dye hepatoma. After discussing, from various points of view, the histogenesis, histologic architecture, and behavior of the hepatoma at the transplantation, *etc.*, we have come to the belief that the Yoshida sarcoma might possibly be a malignant growth derived from Kupffer's stellate cells of the liver. This is only a postulate.

We are at present engaged in a series of studies in an attempt to determine whether Yoshida sarcoma could be artificially produced by treatment planned according to our postulate. Although we have as yet not succeeded in reproduction of the actual Yoshida sarcoma, we established a new strain of transmissible tumor, called the "ascites hepatoma" for the time being.

This "ascites hepatoma" is also an ascites tumor that is artificially transformed from the induced hepatoma of aminoazo dye. Successive transmission of the ascites can be made just as in the case of the Yoshida sarcoma. The hepatoma cells proliferate in the ascites, which accumulates in a large amount, attaining sometimes as much as 30 cc. in one animal. Yet, the cells are never separated from one another as in the case of the Yoshida sarcoma, but rather they are present as cell groups or conglomerates of various shape and size. The surface of each of the conglomerates is covered tightly with an endothelial layer of cells which seem certainly to be derived from Kupffer's cells. Provisionally, therefore, we call these formations "hepatoma islands," and regard them as the fundamental constituents of the hepatoma.

The endothelial cells covering the surface of the hepatoma island are, according to our interpretation, also of neoplastic nature. Our consequent assumption is that if they could be made to separate from the epithelial (hepatic) tumor cells and become isolated in a spacious place like the peritoneal cavity, they would disclose their intrinsic potency of neoplastic proliferation to present a fluid sarcoma of apparent mononuclear origin—the Yoshida sarcoma.

The hepatoma islands grow also on the surface of the peritoneum, resulting in large nodular tumors which invade extensively the tissue of the host. The histologic picture of the tumor tissue is of course consistent with that of hepatoma induced in the liver of rats. The ascites hepatoma has been transplanted, so far, with 100 percent successful "takes." The animals inoculated, a total of more than 100, died without exception in 2 or 3 weeks from tumor invasion.

This handy ascites tumor, being of epithelial origin, very surely transmissible and easily reproducible in every laboratory, will provide a good tool, I think, for various lines of cancer research, especially for chemotherapeutic studies just as Yoshida sarcoma has been utilized. [Our paper of "ascites hepatoma" will appear in a forthcoming issue of the Proceedings of the Academy of Japan.]

NATURE OF THE ORIGINAL YOSHIDA SARCOMA

The original Yoshida sarcoma found in a rat in 1943 (*8*), with which we have been working in Japan for more than 8 years, is an "ascites sarcoma." This should be emphasized among its other various neoplastic characteristics. The tumor cells proliferate in the peritoneal cavity of the rat as individual separate cells. The milky ascites, filled with tumor cells, amounts in a few days to about 5 cubic centimeters or more. At the same time, the cells infiltrate the surrounding tissues of the host animal with the result that massive invading tumors cause the death of the animal. The tumor-bearing animals invariably die very early. Ten to twelve days constitutes the mean survival time, the range of individual variations being very small. This fact seems especially useful when the tumor is devoted to a study of the chemotherapy of cancer.

Transplantation Technique

To obtain the tumor-ascites, we use sharp pointed glass capillaries. One puncture with the capillary tube through the abdominal wall is sufficient to secure approximately 0.1 cc. of the fluid (table 1). Transmission is performed with the same capillary tube containing the ascites, puncturing it into the abdominal cavity of the new host. Its contents are blown into the peritoneal cavity. During the process a droplet of the ascites is available for preparation of smears for microscopic controls.

Table 1.—*Tumor cell content of the ascites*

(1) 0.5–1.0 *million* cells in 1.0 *cu. mm.* ascites
(2) In the routine work are transmitted: 0.01–0.05 cc. ascites = 10–50 *millions* of cells.
In *3–4 days* the abdominal cavity of the inoculated animal presents a state of nearly pure culture of the tumor cells, and dies in 10–15 days on intensive invasion of tumor cells.

Smears of Tumor Ascites

In the routine work, we stain the smear with Giemsa solution. A general view of the smear in low-power magnification is indicated in figure 1. A large number of mitotic figures may be seen in every phase of mitosis. The tumor cells are larger than most normal tissue cells of the rat (fig. 2). The diameter of the cell-body varies from 25 to 35 μ, being occasionally over 40 μ. The cytoplasm colors with Giemsa dyes more intensely than that of normal monocytes of the peritoneal fluid. The nuclei are ovoid or kidney-shaped, equipped with large nucleoli, and are located on one side of the cytoplasm.

The cytocentrum, especially in the younger stages of the tumor cell, can be identified by a light blue area surrounded by lavender-colored cytoplasm. The nucleus is indented on the side of the cytocentrum. The azurophile granules can often be seen, presenting radiation forms.

Neutral red rosettes are beautifully demonstrated, suggesting monocytic origin of the tumor cells. Janus green stained the cells only faintly

(fig. 4). Oxydase reaction is substantially negative. Both fat and glycogen are practically absent. The tumor cells are not, as a rule, phagocytic in nature.

Histology of the Yoshida Tumor

At autopsy of tumor-bearing animals, large solid tumors and extensive infiltration of the omentum, mesenterium, pelvic cavity, and abdominal wall at the site of the puncture are common findings (fig. 5).

Histologically, the tumor is that of the round-cell sarcoma, which resembles the reticulum-cell sarcoma (fig. 3). Invasion of the tumor through the mesenterium into the wall of the small intestine (fig. 6) and infiltration into the liver along the portal vein, resulting sometimes in jaundice (fig. 7), are often seen in advanced stages of this ascites tumor.

Metastasis and Blood Picture

Distant metastasis of the tumor is a rare occurrence. Apparently, it depends on the brief life span of the tumor-bearing animal. In advanced stages of the tumor, *i. e.*, approximately 10 days after the inoculation, tumor cells are found in the peripheral blood. But usually they constitute scarcely one percent of the whole white blood-cell count (fig. 8).

TRANSPLANTATION RATE OF THE ORIGINAL YOSHIDA TUMOR

The number of "takes" of the Yoshida sarcoma is more than 91 percent on the average throughout 322 generations (table 2). We employ for the transplantation only albino rats of mixed breed about 80–100 gm. (3–4 months old) in weight. It was in this mixed breed of albino rat that the original tumor had arisen. The above percentage is regarded as constant from the start of transplantation to the present time. Table 3 indicates the number of "takes" of each generation from the first to the

Table 2.—*Transplantation rate of Yoshida sarcoma from the beginning up to present*

[Intraperitoneal transplantation]

Year	Generation	Number of animals transplanted			Results		Rate (percent)
		Total	Accidents	Valid	+	−	
1943–44 (Nagasaki)	1–38	385	45	340	301	39	88. 2
1944–45 (Tokyo)	39–66	334	108	226	222	4	98. 3
1945	67–87	81	15	66	52	14	78. 8
1946	88–119	134	18	116	108	8	93. 1
1947	120–159	120	7	113	109	4	96. 5
1948	160–196	77	18	59	57	2	97. 6
1949	197–243	117	5	112	110	2	98. 2
1950	244–297	172	11	161	134	27	83. 2
1951 (Sendai)	298–322	97	12	85	77	8	90. 6
Total	1–322	1, 517	239	1, 278	1, 170	108	91. 6

—(Harua Sato)

tenth. A gradual increase in the percentage of "takes" as transplantation passages increase seen in other tumors, is not the case in this tumor. This suggests that the intrinsic nature of the tumor cell, represented by its transplantability, was invariable or fixed at the outset in the original tumor.

TABLE 3.—*Transplantation rate of Yoshida sarcoma in the 1st–10th generations.*

Generation	Number of animals transplanted			Results		Transplantation-rate (percent)
	Total	Accidents, *etc.*	Valid	(+) Death from tumor	(−) Insusceptible	
1	2	1	1	1	0	100. 0
2	8	2	6	4	2	66. 7
3	6	0	6	3	3	50. 0
4	1	0	1	1	0	100. 0
5	6	1	5	5	0	100. 0
6	9	2	7	6	1	85. 7
7	8	0	8	8	0	100. 0
8	10	2	8	8	0	100. 0
9	12	2	10	10	0	100. 0
10	15	5	10	6	4	60. 0
Total	77	15	62	52	10	83. 9

It is demonstrated in the table that the rate in the very beginning of the transplantation was as high as at present. The gradual increase in transplantability by successive animal passages, as generally accepted, is not the case in this tumor.

Another noteworthy fact is that the "takes" will never be one hundred percent, if a sufficient number of animals be taken into consideration. In certain rats, the tumor cells increase in the usual way only for the first few days after the inoculation, and then begin to diminish so that they disappear entirely from the ascites about 1 week later by spontaneous regression of the tumor. Undoubtedly, the frequency of these "non-susceptible" animals determines the rate of transplantation of this tumor. It is implied in this second finding that the determining factor of the transplantability must be traced solely to the host animal, or to its constitutional conditions—but never to the tumor cells.

In our laboratory, Hiroshi Sato (*6*) is inbreeding rats started from parents that have been spontaneously cured of the tumors transplanted (table 4). Among the animals of the F_1 and F_2 generations, nonsusceptible animals are evidently more numerous. The observed material is not yet sufficient, but the result so far obtained strongly suggests that the determining factor of the transplantability is certainly involved in the hereditary constitution of the animal, even though it seems to be of a complicated nature. We are particularly interested in the finding that among such breeds there is an evident increase of those animals in which the tumors grow extraordinarily slowly. Some of them did not die until 2 or 3 months after inoculation; in one extreme case, until 6 months after inoculation, bearing very slowly increasing tumors which, at long last, invaded the host very extensively.

TABLE 4.—*Lowering of the transplantation rate in the inbred offspring of parents insusceptible to Yoshida sarcoma*

	Total number of animals transplanted	Susceptible				Insusceptible [3]	
		Regular number	Growth [1] percent	Irregular number	Growth [2] percent	Number	Percent
F_1	229	122	53. 3	48	20. 9	59	25. 8
F_2	138	59	42. 8	34	24. 6	45	32. 6
F_3	5	0	0	1	20. 0	4	80. 0

—(Hiroshi Sato)

[1] Animals died of tumor invasion in the regular course of 10–20 days, averaging 12 days.
[2] It needed a long time, varying from 1 to 6 months, before the animals died of tumors which had grown slowly. Those animals in which the tumor cells increase very slowly, though they do not cease, are also occasionally met with among usual mixed-bred rats, but at very low rate being estimated less than 5 percent.
[3] It was confirmed after 6 months that the animal bodies were entirely free from tumor cells.

Animals of the Wistar strain have shown in our laboratory a considerably small number of takes (table 5). But, it must be added that other investigators in Japan recorded a higher percentage, approximately 80 percent, employing a larger number (over 100) of animals than we did.

TABLE 5.—*Transplantation rate in the rats of the Wistar strain*

Number of animals transplanted			Result	
Total	Died of interposed causes	Effective	+	−
32	4	28	10 (36 percent) [1]	18 (64 percent)[2]

—(Hiroshi Sato)

[1] Died of tumor invasion in regular course of 10–15 days.
[2] Including cases of irregular progression, which survived more than 30 days bearing very slowly increasing tumors, some of them alive at present.

TRANSPLANTATION OF THE ORIGINAL YOSHIDA SARCOMA BY A SINGLE CELL

The Yoshida ascites tumor can be transplanted so that the exact number of cells in the inoculum may easily be known. At first, we attempted to estimate the minimal number of cells necessary for the transmission of the tumor. Reducing gradually the number of cells, we determined at last that a single tumor cell is sufficient for effective transmission. The method of single-cell transplantation carried out by Ishibashi (*2*) in our laboratory is as follows:

The tumor ascites is diluted to such a degree that a droplet of this diluted ascites may contain only one, a few, or no tumor cells. The degree of the dilution differs by different intensity of population of tumor cells in the original tumor ascites, ranging from about 50,000 to 100,000 times. Small droplets of a properly diluted tumor ascites are placed in rows on a cover glass which is bridged over the moist chamber with the droplets hanging. Each droplet is examined under the microscope for its content of tumor cells. A droplet containing only one cell (fig. 9) is sucked into the micropipette by the aid of a micromanipulator, and

then some of the dilution medium is additionally sucked into the pipette in order to make the total amount of fluid content of the pipette sufficient for the inoculation technique—about 0.01 cc. The micropipette is punctured through the abdominal wall of a normal rat and its content carrying a single tumor cell is blown into the peritoneal cavity of the animal.

Ishibashi at first used plain physiologic NaCl solution or Tyrode solution as the dilution medium, and did not succeed in the one-cell transplantation. He then tried to milden the medium by adding to it the normal peritoneal fluid, using the following procedure: First, he injected 15 to 20 cc. of the physiologic NaCl solution into the peritoneal cavity of a normal rat, then aspirated 5 to 7 cc. of it after 30 minutes and centrifuged it for 60 minutes with the frequency of 3,000 cycles per minute. The supernatant liquid, being completely cell-free, was used for the dilution of the tumor ascites. He obtained good results with this buffered dilution medium.

At the moment of the inoculation with one cell, the tumor ascites within the pipette is estimated to be diluted as high as several million times. Therefore, the injuring effect of the suspension medium upon the single cell may play considerable influence on the result of the inoculation.

Hosokawa (*1*) re-examined in our laboratory the Ishibashi experiment and inoculated 150 albino rats of mixed-breeding with a single cell in each animal. Positive "takes" were obtained in 69 animals.

Hosokawa (*1*) made a further test in order to see what percentage of single cells sucked up into the capillary tubes actually got into the animal body upon inoculation. He sucked up into the micropipette a droplet containing one cell, and blew it out upon the object glass so that a Giemsa-stained smear could be prepared. He repeated the same procedure 100 times, and attained a fairly good result. In 83 cases, the tumor cell was found on the glass. Accordingly, we regarded 125 as effective out of 150 cases of inoculation, as is given in table 6, indicating that the one-cell transplantation is successful in 55 percent of the cases (table 6).

TABLE 6.—*Transmission with a single tumor cell*

Cases of inoculation		Result		
Total number	Effective	+	−	Percent
150	125	69	56	55. 1

—(K. Ishibashi and K. Hosokawa)

The 69 tumors derived from single cells were the same as the usual tumors derived from millions of cells, except that they required in every case about 10 days more for the development of a pure culture state of tumor cells, while growth of ascites tumors derived from millions of cells required much less time. It may be said, therefore, that all tumor

cells of the Yoshida sarcoma are the same in innate character, so far as they have potency to increase, in spite of different appearance in the stained preparations.

The concept seems to be still current that a malignant tumor is a population of various sorts of stem cells differing from one another in the grade of malignancy or "anaplasia." However, this question is not settled. The above observation does not support this idea.

Concerning the virus problem of the Yoshida sarcoma, it may be noteworthy to add that all attempts to transmit the tumor with materials microscopically confirmed to be free of tumor cells in the same way as the one-cell transplantation were always negative. Moreover, in the cases of cell-free materials, the ascites was diluted only 1 to 5 times, while in the one-cell cases it was diluted more than 50,000 times. If there was any free virus in the ascitic fluid, a rather high percentage of takes with nondiluted cell-free materials should be expected. But this is not the case, and the result agrees with many experiments that failed to transmit the tumor with the Chamberland filtrates of the ascites.

CHROMOSOMES OF THE TUMOR CELLS

A large number of mitoses may be observed in smears of Yoshida sarcoma. This shows the very rapid growth of the tumor.

By the aid of the phase-contrast microscope, we attempted to measure the entire course of time required for the completion of a single mitosis of this tumor cell (*9*). According to the results indicated in table 7, it may be safe to say that the average time in which mitosis accomplishes its entire course is about an hour or less.

TABLE 7.—*Course of time of the mitosis of Yoshida sarcoma cells*

[Phase-contrast-microsopic observation]

Case	Phases				Total
	Prophase	Metaphase	Anaphase	Telophase	
1	8′30″(+x_1)	4′30″	3′00″	31′00″	45′00″
2	16′00″(+x_2)	4′30″	2′30″	32′10″	55′00″
3	5′00″(+x_3)	7′30″	9′10″	14′50″	36′30″
4	6′30″(+x_4)	7′00″	2′00″	34′00″	49′30″
5	6′30″(+x_5)	9′00″	3′00″	31′00″	49′00″
Average					47′00″+x

—(Harua Sato and Akira Atsumi)

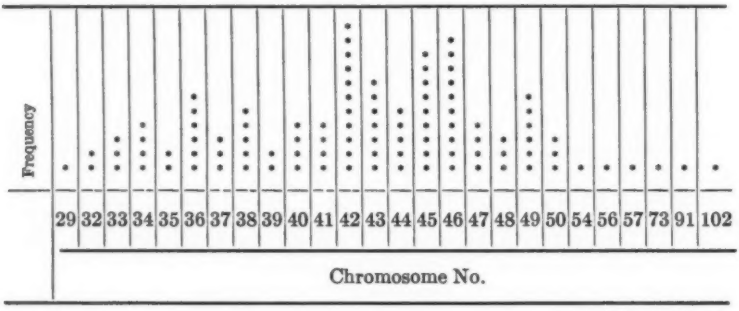
NOTE.—In every case the observation was started from a moment during the prophase, before which some lapse of time must have passed. This unmeasured time is indicated with x that varies according to cases from x_1 to x_5. Of course the x cannot be very large. Therefore it may be safe to estimate the average time in which a mitosis completes within 1 hour.

Harua Sato (*5*) in our laboratory has studied the chromosomes, employing tumors derived from one cell so that the variety of findings could be interpreted in a rather simple way. On 100 nuclear plates, he examined the number of chromosomes and found that it varied with fairly wide range (table 8). Forty-two, the regular chromosome number of the

germ cell of the rat, makes the peak of the variation curve. But only 11 out of 100 nuclei had this number. The majority of the nuclei showed irregular numbers, and fluctuated from 32 to 50.

Sato described V-shaped chromosomes (fig. 10). The V-shaped ones differ in size, being larger or smaller. The frequency of V-chromosomes and the relation of their appearance to the total chromosome number of the cell are indicated in table 9. They appeared in 42 of 100 nuclear plates, the remaining 58 having none of them at all. The number of chromosomes of nuclear plates containing V-shaped ones were subject to fairly extensive variation.

TABLE 8.—*Graphic exhibition of the variations of chromosome number of Yoshida sarcoma cells*



—(Harua Sato)

NOTE:—42 is the regular number of chromosomes in the rat.

TABLE 9.—(1) *Frequency of the V-shaped chromosomes. [Among 100 nuclear plates 42 had V-shaped ones (42 percent); 58 had none of them]* (2) *Relation of the appearance of V-shaped chromosomes to the chromosome number of the tumor cells*

Chromosome No.	35		36–40		41–45		46–50		51		Total	
V-chromosome	(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)
	2	10	4	16	21	15	12	14	3	3	42	58

—(Harua Sato)

The V-shaped chromosomes appear to be somewhat striking. However, considering Sato's findings, it would seem to be far-fetched to attribute any special significance to them in the genesis of tumor cells. I agree with Sato's statement that his findings very likely indicate that the V-chromosomes can appear in any Yoshida sarcoma cell, depending on some yet unknown conditions. In this connection, it is important to note that he has studied cells of tumors of single-cell derivation.

APPLICATION OF THE YOSHIDA SARCOMA TO STUDIES ON CHEMOTHERAPY OF CANCER

According to the following plan, we are testing various chemicals supplied by Prof. M. Ishidate and Dr. Y. Sakurai of Tokyo University. Through their cooperation, first in 1949, we have to some extent systematized our research on the chemotherapy of cancer.

STEPS OF THE "SCREENING-TEST" OF CHEMICALS EMPLOYING YOSHIDA SARCOMA ANIMALS

A

(1) Determination of M. T. D. (maximal tolerated dose).

(2) Intraperitoneal injection of rats, bearing about 3 to 4 day-old Yoshida sarcoma, of a dose close to M. T. D. of the chemical tested.

(3) Examination of the tumor-ascites of each animal at 1, 3, 6, 12, and 24 hours after injection of the chemical.

NOTE:—*A small amount of the ascites (0.05 cc. or so) obtained by means of puncture with glass capillaries is sufficient. The animals tolerate easily the repetition of punctures at short intervals as here prescribed.*

Procedure for examination of the tumor-ascites:

In fresh materials:

(a) Number of tumor cells in 1 cu. mm. of ascites.
Attention is paid to the extent of decrease in the number of cells after treatment, as well as to increase in the number of cells during recovery.

In Giemsa stained smears:

(b) Evaluation of changes in nuclei, cytoplasm, and chromosomes of the tumor cells. Changes in the total number of mitoses are considered.

(c) Approximate estimation of the proportion of damaged to intact tumor cells.

(d) Mode of reaction of the normal cells in the peritoneal cavity.

Note on the cellular reactions in the ascites:

Before the injection of chemicals, the tumor presents a state of almost pure culture of tumor cells in the ascites. Neutrophils are essentially absent. Following the injection, a prompt response of the neutrophils is a common occurrence. If destruction of the tumor cells takes place, it is followed by an increase of monocytes which actively phagocytize the detritus. The grade of these reactions differs with different chemicals, suggesting accompanying toxicity of the substance tested upon the normal tissue cells.

(4) After 24 hours, if the animal survives, the ascites is examined once a day until its death.

Whether or not a prolongation of life is caused by a single dose? Cause of death and grade of tissue invasion of the tumor cells are examined by autopsy.

B

(1) Substances that passed the screening "A" are further examined by repeating administration of minimal effective doses in order to determine whether they are capable of causing a recovery from the disease, or at least a significant prolongation of life of the tumor-bearing animals.

(2) If the effect of a substance is definite by direct intraperitoneal application, then the effect is examined further by subcutaneous administration, *etc.*

Recently, in a series of studies with compounds related to nitrogen mustard, the N-oxide derivative of the substance, methyl-bis-(β-chlorethyl)-amine N-oxide (MBAO) revealed an interesting fact.

According to Ishidate and Sakurai, who prepared the compound after the method of Stahmann and Bergmann, MBAO is remarkable at least in two points: 1) MBAO is almost completely hydrophile, while MBA (nitrogen mustard) is lipophile, and 2) the rate of the chlor liberation of MBAO in aqueous solution is remarkably low. The toxicity of MBAO is lowered by about 10 times that of the MBA as such, without losing the essential efficacy; *i. e.*, the range of safe administration is remarkably extended in the N-oxide compound.

The details of animal experiments carried out by us (*3*) are indicated in table 10. The results so far obtained are as follows: 5 of 20 tumor-bearing animals treated with this substance have recovered from the tumor, while the survival time of the remaining 15 rats was prolonged by two to three times that of the control animals.

TABLE 10.—*Result of repeated injections of nitrogen mustard N-oxide (MBAO*)*

	Animal No.	Single (mg.)	Dose (number)	Total (mg.)	Survival (days)	Causes of death	Remarks
Intraperitoneal injection	5811	1. 5–2. 0	4	7. 5	64	Pneumonia	Tumor cells ceased.
	5812	2. 0–1. 5	5	7. 5	→		85 days (on July 10, 1951).
	5813	1. 5–2. 0	5	7. 5	→		Do.
	5814	1. 5–2. 0	5	7. 5	→		Do.
	5854	2. 0	3	6. 0	24	Debility	Tumor cells decreased.
	5855	1. 0–2. 0	9	13. 0	→		66 days (on July 10, 1951).
	5856	1. 0–2. 0	4	7. 0	37	Tumor invasion.	
	5857	1. 0–2. 0	4	7. 0	28	do	
	5858	1. 0–2. 0	5	8. 0	38	Debility	Tumor cells decreased.
	5859	1. 0–2. 0	4	7. 0	30	do	Do.
	5908	. 8–1. 0	3	2. 8	21	do	Do.
	5916	1. 0	5	5. 0	30	Tumor invasion.	(+ debility).
	5917	. 8–1. 0	3	2. 8	18	Debility	Tumor cells ceased.
	5918	1. 0	4	4. 0	28	Tumor invasion.	
	5919	1. 0–1. 5	5	6. 0	32	do	
Subcutaneous injection	5866	1. 0–2. 0	9	13. 0	→		60 days (on July 10, 1951).
	5867	1. 0–0. 2	3	5. 0	25	Tumor invasion.	
	5868	2. 0	2	4. 0	30	Debility	Tumor cells decreased.
	5906	. 8–1. 5	3	3. 3	15	Intoxication	
	5907	1. 0	1	1. 0	17	Tumor invasion.	(+ debility).

—(K. Kobayashi)

*Methyl-bis-(β-chlorethyl)-amine N-oxide (MBAO); the compound was prepared by Ishidate and Sakurai, modifying the method of Stahmann and Bergmann published in 1946 (J. Org. Chem.). M. T. D.: 3 mg., *i. e.*, 10 times the dose of MBA as such.
→Animals have completely recovered from tumor.

Coincident with the inhibiting effects, remarkable changes were also observed in mitosis of tumor cells. As early as 3 hours after administration of the chemicals, the total number of mitoses was evidently decreased, and continued to decrease until at 12 hours the mitotic figures in the ascites almost completely disappeared. And then they began gradually to increase, regaining in 48 hours almost the initial level (table

11). It is worthy to note that for this long-range response a single dose of 1.0–2.0 mg. (1/3–2/3 M. T. D.) of the substance was sufficient. Moreover, the subcutaneous application of the substance caused the same effect as the direct intraperitoneal injection upon the tumor-ascites.

TABLE 11.—*Number of mitoses among 2,000 tumor cells after intraperitoneal injection of nitrogen mustard N-oxide*

Animal No.	Dose (mg.)	Mitotic phase	Before injection	Hours after injection				
				3	6	12	24	48
5809	2	Pro	15	2	1	0	3	8
		Meta	7	2	1	0	6	14
		Ana	5 (33)	0 (7)	0 (2)	0 (0)	4 (17)	3 (28)
		Telo	6	3	0	0	4	3
5846	1. 5	Pro	7	1	0	0	0	3
		Meta	8	2	1	0	4	14
		Ana	12 (40)	2 (8)	0 (3)	1 (2)	2 (6)	5 (24)
		Telo	13	3	2	1	0	2
5811	2	Pro	11	3	1	0	2	3
		Meta	9	4	1	0	2	6
		Ana	7 (35)	4 (16)	1 (3)	0 (0)	0 (4)	4 (14)
		Telo	8	5	0	0	0	1
5916	1	Pro	7	2	1	1	3	4
		Meta	11	3	2	2	0	18
		Ana	5 (36)	3 (10)	0 (3)	1 (5)	3 (9)	13 (39)
		Telo	13	2	0	1	3	4
5918	1	Pro	16	5	1	0	1	5
		Meta	18	6	1	1	4	20
		Ana	6 (53)	2 (21)	0 (4)	0 (1)	1 (9)	5 (35)
		Telo	13	8	2	0	3	5
5919	1	Pro	8	4	0	0	0	2
		Meta	13	2	1	2	3	20
		Ana	5 (40)	4 (14)	0 (3)	0 (2)	1 (6)	5 (31)
		Telo	14	4	2	0	2	4

—(K. Kobayashi)

The mitotic figures after 12 hours indicated interesting changes that we can trace in detail in a series of the smear preparations of the ascites. Photographs (reproduced in figs. 11–14) reveal: scattering of lacerated chromosomes, coagulation of chromosomes, and breaking-up of chromosomes into irregular groups and chromosome bridges. Inconsistency of cytoplasmic and nuclear division is a peculiar finding. The separation of the cell body advances independently and results in forced tearing of the nuclear mass (figs. 15–18).

A normal figure of mitosis is difficult to find after treatment with MBAO. Hence, this chemical agent seems to exert a special influence upon the mitotic process of the tumor cell. This interpretation is supported by the fact that 72 or 96 hours following administration of the chemical the ascites consists of enormous tumor cells, probably in polyploidy (figs. 19–26). These enormous cells usually have huge nuclei (probably popyploid), or they may possess many separate nuclei and scattered nuclear fragments that are apparently products of the scattered chromosome groups. The majority of tumor cells of this stage belong to this type. Karyolysis and cytolysis of giant tumor cells are common.

It is evident that these abnormal, enormous cells can neither survive long nor proliferate. In fact, hand in hand with the appearance of these abnormal cells, there also appears in the ascites a large number of monocytes which phagocytize the debris of the destroyed tumor cells (fig. 19). In a few days after that, the ascites becomes almost free of tumor cells.

The subcutaneous nodular tumors of Yoshida sarcoma also respond to the administration of MBAO. They regularly show striking regression in response to one or two doses of the substance. K. Kajiwara is carrying on experiments in this line.

All things considered, it may be said that our findings are very encouraging for further research on this and other related chemical compounds.

ADDENDUM

Recent Trends of Cancer Research in Japan

Waro Nakahara,

President of the Japanese Cancer Association and Director of the Cancer Institute of the Japanese Foundation for Cancer Research, Tokyo, Japan.

My esteemed colleague, Professor Tomizo Yoshida, requested that I prepare a short note on the recent trends of cancer research in Japan. In writing the following lines, I have gone over anew the papers presented at the last few annual meetings of the Japanese Cancer Association, a nation-wide and the only scientific society devoted to cancer research in Japan. I have also taken into consideration the recent scientific reports of the Cancer Research Group, supported by grants-in-aid (Scientific Research Grants) of the Ministry of Education of the Japanese Government. This "Group" is made up of some twenty active workers in various fields of cancer research, and its work may be regarded as representing the "top layer" of cancer research in Japan.

Because of the background of the majority of active workers, cancer research in Japan has long been dominated by anatomo-pathologic studies. In recent years a shift toward more experimental and biochemical studies has taken place, but not to the extent now prevailing in the United States. It may be surprising to American colleagues to know that some 35 percent of the papers presented at 1951 meeting of the Japanese Cancer Association were on histopathologic studies on human tumors, while biochemical papers amounted to less than 20 percent of the total. This is in a striking contrast to the situation of the American Association for Cancer Research, where 40 to 50 percent of the papers presented are devoted to biochemical subjects and histopathology of human tumors is conspicuous by its absence. In this connection, it should be pointed out that there is a strong tendency among Japanese pathologists to present their papers on tumor pathology at the Cancer Association, instead of at the Pathological Society, with the result that papers on tumors at the Japanese Pathological Society are disproportionally small in number.

An extraordinary situation in the recent cancer research in Japan is the use of "Yoshida sarcoma" of the rat as material for all kinds of tumor

studies. No one type of tumors has ever enjoyed so great a popularity as this sarcoma in any country and at any period in the history of cancer research. It is no exaggeration to state that nearly 30 percent of the total cancer literature in Japan during the past few years is related to this Yoshida sarcoma.

The Yoshida sarcoma, first discovered and established as a transplantable strain by Professor Yoshida in 1943, was so named at the suggestion of Professor Kinoshita by the Japanese Cancer Association in 1948. It is an ascites sarcoma, probably of monocytic origin. Because the sarcoma cells proliferate free in the peritoneal fluid, it is an excellent material for cytological observations, and much work has been done along these lines. Cell division and amitosis have been observed in the living condition. Finer cytological studies on mitochondria and Golgi apparatus have been made. The establishment of the chromosomal characters of this sarcoma may be regarded as a very important contribution. Dr. Sato, in Yoshida's laboratory, especially, worked out the chromosomal character of this sarcoma based on observation on the sarcoma cells derived from a single cell. Dr. Ishibashi, also in Yoshida's laboratory proved that this sarcoma can be transplanted by the implantation of a single sarcoma cell—another important finding.

Practically all the aspects of tumor biology have been reinvestigated using this sarcoma: Behavior in tissue culture, transplantability in various strains of rats and animals of different species, histologic and immunologic reactions involved in these cases of transplantation, transplantability to chick embryos, filtration and centrifugation experiments, resistance of the sarcoma cells to low temperature, heat, X-rays, supersonic waves and other physical agents and all kinds of chemical substances, endocrinological relations involving estrogenic and thyroid hormones, respiration and glycolysis *in vitro*, changes in enzyme actions and serum protein in the sarcoma-bearing animals, *etc.*

These extensive investigations placed the Yoshida sarcoma in line with the usual mammalian tumors so far studied. The existence of virus, or causative agent separable from sarcoma cells, has not been demonstrated.

The Yoshida sarcoma is also a very convenient tumor to be used in screening tests for chemotherapy experiments, and much work is being done according to a plan for such tests formulated by Professor Yoshida.

Chicken sarcoma, once a popular subject in Japan, is now receiving little attention, largely because, I think, of the prohibitive cost of chickens necessary for experiments.

Azo dye carcinogenesis and its inhibition are still being studied, but recent progress has been insignificant; nor has there been a discovery of a new carcinogenic substance.

Before bringing this short note to a close I wish to refer to the subject of my own interest. Dr. Fukuoka and I isolated in 1949 a thermostable, non-heat coagulable, water soluble and alcohol precipitable protein-like substance from human malignant tumors, which has the property of markedly lowering the liver catalase of mice, and called it "toxohormone."

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This is the characteristic toxic substance that accounts for the well-known low liver catalase in tumor-bearing animals. We later obtained evidence showing that toxohormone may have the property of binding iron, so interfering with the synthesis not only of catalase but also of all the biologic systems containing iron as the prosthetic group. Several papers appeared just lately in Japan, confirming and extending the original observation, chief among them being the demonstration of a similar catalase-lowering substance in the urine of cancer patients (Professor Nakagawa). The discovery of toxohormone may prove to be a sufficient reason for the revival of interest in the study of cancer cachexia—(July 1951).

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Plate 86

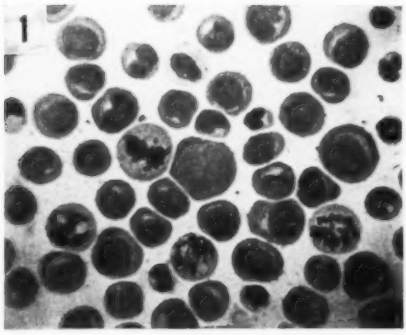
Figure 1.—A general view of the smear of Yoshida sarcoma ascites stained with Giemsa and showing many mitotic figures.

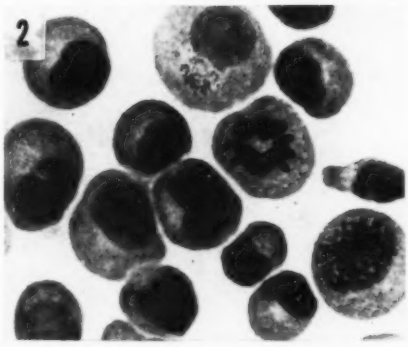
Figure 2.—A higher power magnification of figure 1.

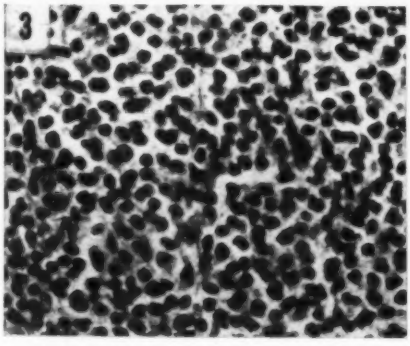
Figure 3.—Histological picture of a solid tumor of Yoshida sarcoma.

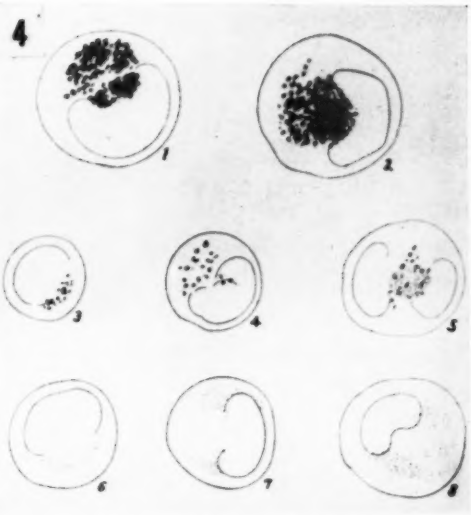
Figure 4.—Cells 1–5: neutral red rosette of Yoshida sarcoma cells. Cell 5 is binucleated. Cells 6–8: Janus green granules.

Figure 5.—Autopsy picture of an animal with a Yoshida sarcoma. Formation of large solid tumors in the omentum and other locations. The ascites is removed.









Yoshida

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PLATE 87

FIGURE 6.—Infiltration of tumor cells through the mesenterium into the wall of the small intestine.

FIGURE 7.—Infiltration into the periportal tissue.

FIGURE 8.—Picture of the heart's blood of an 8-day-old animal with Yoshida sarcoma. One of two tumor cells is in mitosis.

FIGURE 9.—A tumor cell contained in a droplet of the Yoshida sarcoma ascites diluted by about 200,000 times.

FIGURE 10.—Nuclear plates of Yoshida sarcoma cells containing Y-chromosomes (black) compared with those having none of them (Haruo Sato).

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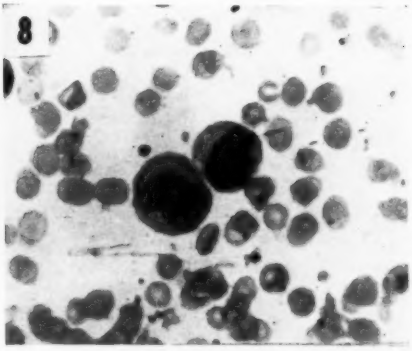
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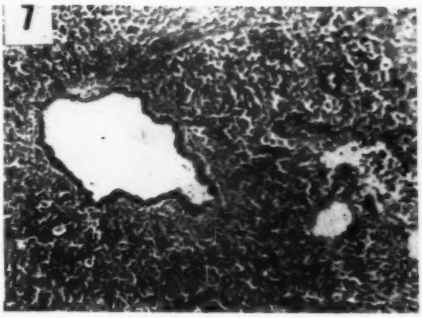
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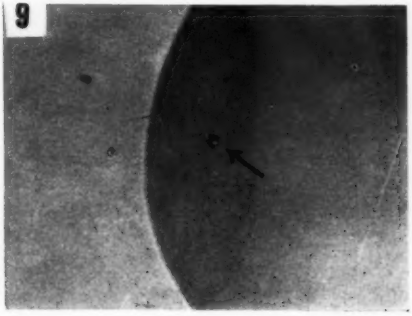
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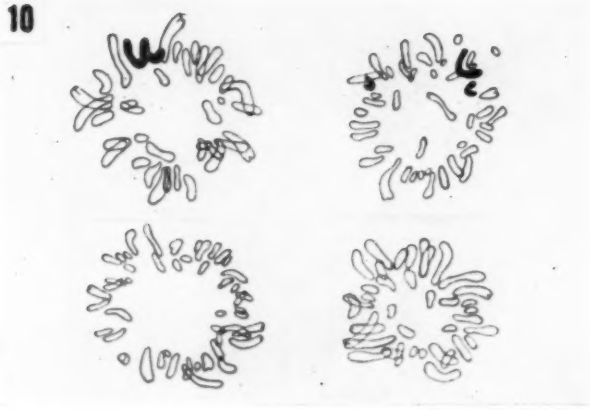
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Plate 88

(Twenty-four hours after the administration of nitrogen mustard N-oxide; findings in the smear of Yoshida sarcoma ascites stained with Giemsa)

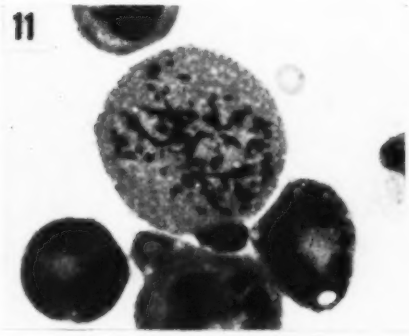
Figure 11.—Scattering of lacerated chromosomes.

Figure 12.—Coagulation (left) and breaking-up (right) of chromosomes into irregular groups.

Figure 13.—Breaking-up of chromosomes into irregular groups.

Figure 14.—Chromosome bridges.

Figure 15–18.—Inconsistency of cytoplasmic and nuclear divisions. In figures 15 and 16 independently advancing division of cytoplasms is illustrated. Lagging nuclear divisions. Figure 17 shows forced tearing of nuclear substance by already completely divided daughter cells. Figure 18 shows fine nuclear string between two daughter cells, apparently the end stage of the process indicated in the foregoing pictures.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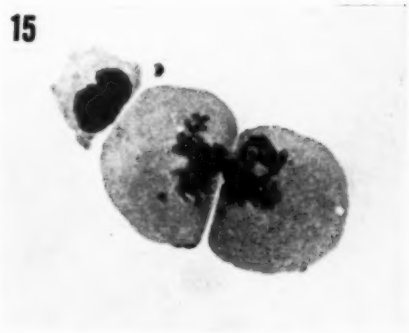
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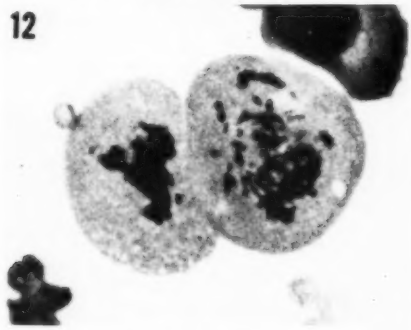
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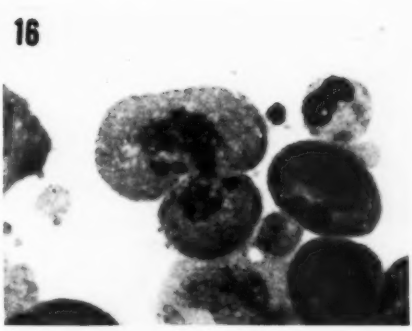
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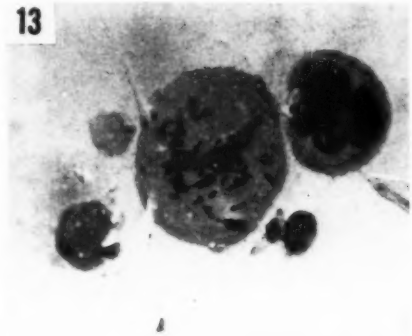
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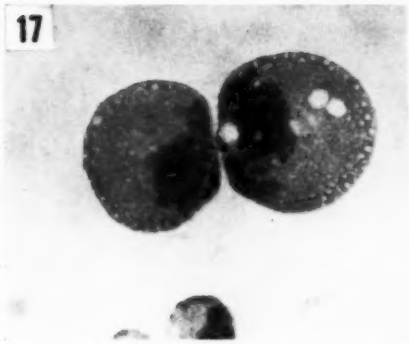
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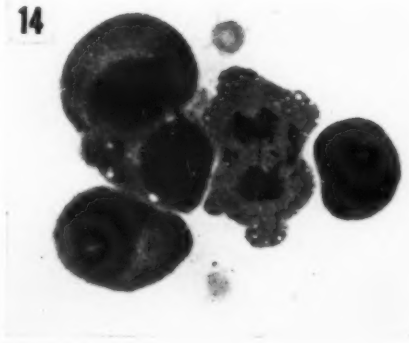
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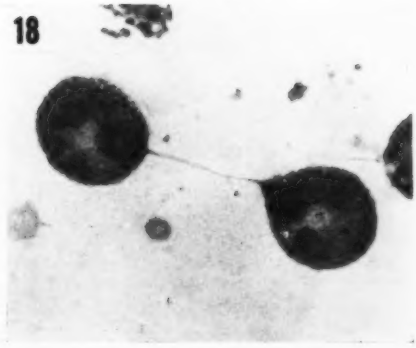
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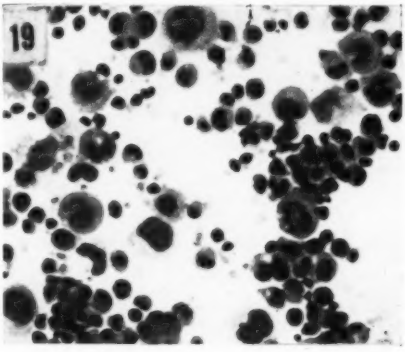
PLATE 89

(Forty-eight hours after the administration of nitrogen mustard N-oxide; findings in the smears of the Yoshida sarcoma ascites stained with Giemsa.)

FIGURE 19.—Almost all tumor cells are of enormous size. Numerous normal monocytes are seen which actively phagocytize the debris of degenerated tumor cells.

FIGURES 20–25.—Enormous cells, segregated nuclei, and nuclear fragments are illustrated.

FIGURE 26.—Degeneration of the enormous cells.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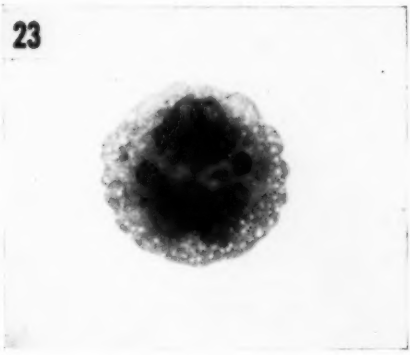
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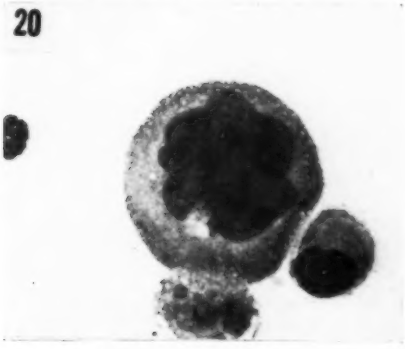
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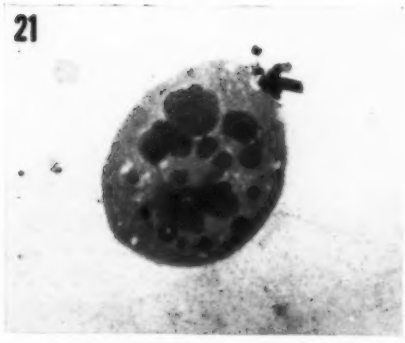
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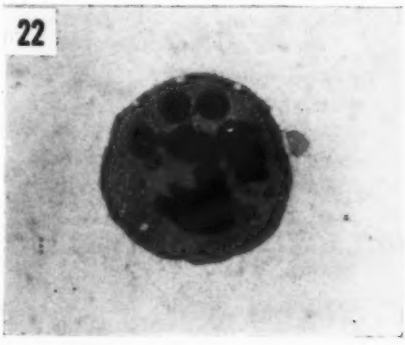
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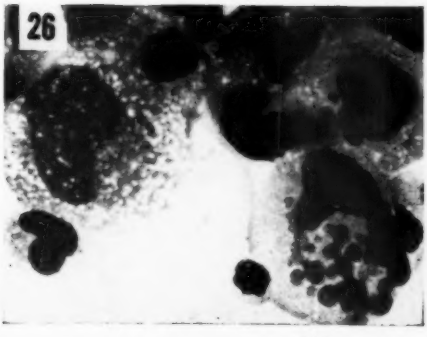
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STUDIES ON THE OXIDASE ACTIVITY OF NORMAL AND MALIGNANT LYMPHOID TISSUES [1]

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The object of this study was to investigate the terminal oxidative enzyme system of human lymphoid tissue under the stress of disease in general, and of Hodgkin's disease in particular. Our attention was focused on the terminal oxidative systems because the work of Warburg (*15*), Greenstein (*2*), and Raskelley (*12*) indicated that the content of cytochrome oxidase and cytochrome c in tumor tissue is low. The question as to whether Hodgkin's disease is inflammatory or cancerous in nature being still unresolved, it was thought that data concerning the terminal oxidative system of lymphoid tissue might throw some light upon this aspect of the Hodgkin's disease problem. According to present concepts (*8*), the terminal oxidative system comprises the following: cytochrome oxidase (respiratory enzyme or indophenol oxidase), cytochrome c, possibly cytochromes a and b, and succinic dehydrogenase.

MATERIALS AND METHODS

With the aid of simple Warburg manometers, the oxidase activity of the various tissues was determined by measuring the rate of oxygen uptake in the presence of hydroquinone and succinate as substrates and of cytochrome c as a mesocatalyst [Keilin and Hartree (*5*)].

Tissues used in these experiments were of human and animal origin. The human material was spleen, lymph node, thymus gland, tonsil, and white blood cells. The animal tissues were heart and liver of the rat, spleen of the pig, thymus gland and heart of the calf, and gastrointestinal mucosa of the rabbit. Two lymph nodes were obtained from two autopsies of man, and cytochrome oxidase activity determined 12 hours after death. Human thymus glands obtained from two other autopsies were analyzed 48 hours after death. Storage conditions were the same as for biopsy material. Animal tissues were obtained either from animals sacrificed in the laboratory immediately before the experiments, or from fresh

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[2] Department of Chemistry, Polytechnic Institute of Brooklyn, Brooklyn, N. Y.
[3] Supported in part by grants from the American Cancer Society, and the Damon Runyon Memorial Fund.

slaughterhouse material, the latter tissues being kept on ice during transportation to the laboratory where it was stored at 1° C. until used.

In order to obviate differences in the rate of diffusion of substrate into sliced tissue as a limiting factor, and also because of the difficulties involved in preparing satisfactory slices from tissues such as lymph nodes, tissue homogenates were used in all experiments. In preparing homogenates, glass and stainless steel homogenizers were tried. Distilled water was added and the tissue ground to a fine pulp. The steel homogenizer was unsatisfactory because the friction between the mortar and pestle resulted in the contamination of the homogenate with fine metal particles. A semi-micro glass homogenizer, the mortar of which had a grinding volume of 2 ml., proved satisfactory for such soft tissue as liver but was inadequate for lymph nodes which had become fibrous. The all-glass homogenizer which proved most useful was that of Potter and Elvehjem (*10*). During preparation of the homogenates the homogenizer was immersed in ice water. Microscopic examination of smears made from several homogenates showed complete destruction of cellular structure.

The cytochrome c which was added to the homogenates was in the form of highly purified ferricytochrome c[4] from heart muscle of the horse.

The hydroquinone[5] used as substrate was of C. P. grade and used without further purification, except for one experiment in which vacuum-sublimed hydroquinone was used.

Sodium succinate[6] was of U. S. P. quality and was used without further purification. The experiments were carried out at 25.2° C. with air as the gas phase. The shaking rate was 180 oscillations per minute. From 1 to 3 ml. of the homogenate, depending upon its activity, were pipetted into the main chamber of a 15-ml. conical reaction vessel, the center well of which contained 0.25 ml. of 10 percent KOH. To the homogenate were added 0.5 ml. of 0.5 M phosphate buffer, pH 7.1, and 0.5 ml. of a cytochrome c solution containing as a rule 1.0 milligram of cytochrome c. The side bulb contained 0.25 ml. of 0.1 M hydroquinone or sodium succinate solution. In the case of hydroquinone, another vessel containing no tissue was used as autoxidation control. In general, the determinations on a given tissue were performed in duplicate. The average final concentration of hydroquinone and sodium succinate was $7.9 \times 10-3$ M based on an average total volume of 3.5 ml. per flask, while the cytochrome c concentration was usually $1.8 \times 10-5$ M.

Dry weight of tissue was determined by pipetting aliquots of the homogenate into tared porcelain crucibles and drying for 24 hours at at 110° C. Oxygen consumption (Q_{O_2}) for individual reaction vessels was calculated as follows [*cf.* Dixon (*1*)]:

$$Q_{O_2} = \underset{\text{(for oxygen)}}{\overset{(K)}{\text{(vessel constant)}}} \times (\text{mm. } O_2 \text{ uptake}) \times \left(\frac{60}{\text{duration of experiment in minutes}}\right) \times \left(\frac{1}{\text{dry weight of tissue in mg.}}\right)$$

[4] Obtained from the Hygrade Laboratories, Sigma Chemical Co., and the Wyeth Institute of Applied Biochemistry.

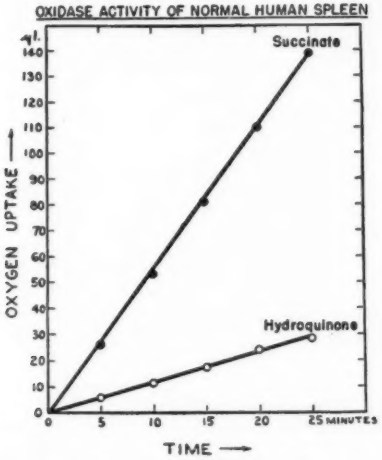
[5] Obtained from Fisher Scientific Co.

[6] Obtained from the Mallinckrodt Chemical Co.

The value for K ranged from 1.56 to 1.64 for the various manometer vessels employed.

OBSERVATIONS

A representative manometric experiment performed on human spleen, removed during partial gastrectomy for peptic ulcer, is shown in text-figure 1.



TEXT-FIGURE 1.—Human spleen from a patient with peptic ulcer, histologically normal.

Similar curves were obtained from experiments performed on tissue homogenates from organs other than spleen (lymph nodes, thymus glands, *etc.*), from normal animals, and from cancerous and noncancerous tissue of man. It will be noted that the rate of oxygen uptake is linear throughout the period of observation (25 minutes). This indicated constant enzymatic activity and the presence of an excess of substrate under these conditions. A linear curve was observed in several instances where the duration of the experiment was extended to 1 hour. The effect of varying the experimental conditions on the rate of oxygen uptake will be discussed below.

The only human spleen that could be considered normal histologically was removed during gastrectomy from a patient with gastric ulcer. This showed a low oxidative activity toward hydroquinone ($Q_{O_2}=0.29$) and a somewhat higher activity with succinate as the substrate ($Q_{O_2}=0.85$). Spleen from a normal pig was found to possess a much greater oxidase activity than this human spleen (table 1). Oxidase values obtained on various tissues from several species are listed in table 1.

TABLE 1.—*Oxidase activity of various normal tissues*

Species	Tissue	Number of specimens	Average	
			Q_{O_2} (hydroquinone)	Q_{O_2} (succinate)
Man	Spleen	1	0. 26	0. 86
Rat	Spleen	1	10. 0	7. 4
	Heart	3	21. 9	31. 1
	Liver	1	----------	14. 5
Pig	Spleen	2	4. 55	3. 26
Beef	Skeletal muscle	1	. 2	. 4
Calf	Thymus	2	. 88	3. 05
	Heart	2	17. 6	17. 1
Rabbit	Gastrointestinal mucosa	1	6. 7	3. 8

We were unable to obtain normal human lymph nodes for these experiments. Thymus gland of the calf was the tissue most closely approximating an organ composed predominantly of lymphocytes which we could obtain in quantities sufficient for the experiment. Its Q_{O_2} activity toward hydroquinone substrate was 0.88; toward succinate substrate, $Q_{O_2}=3.05$. The values for thymus gland obtained at autopsy from a 9½-day-old infant diagnosed as *status lymphaticus* was $Q_{O_2}=0.46$ for hydroquinone. In this latter experiment, succinate was not employed as a substrate.

It will be noted from table 1 that the oxidase activity of heart and liver tissue of the rat (toward succinate) is of a higher order of magnitude than is that of other tissues (skeletal muscle, spleen, mucosa, thymus gland) of other species. The same is true of the heart of the calf. Another point of interest concerns the relative activity of the various tissues toward hydroquinone and succinate; in the case of human spleen and thymus of the calf, this ratio is approximately 1:3, whereas in the spleen of the pig and the gastrointestinal mucosa of the rabbit, the ratio is reversed (1.4:1 and 1.7:1, respectively).

Study of the cytochrome oxidase activity of the homogenates prepared from both spleens and lymph nodes of patients with various diseases (tables 2 and 3) indicates that the activity is low, but no definite correlation is evident. Thus, the Q_{O_2} for hydroquinone as a substrate varied from 0 to 1.36 for lymph-node homogenates and from 0.20 to 2.69 for spleen homogenates; for succinate as a substrate the Q_{O_2} values were 0.7 to 1.51 for lymph-node material and 0.23 to 3.78 for spleen material. These variations were considered to be actual and not the result of technical difficulties since duplicate determination for the individual specimens showed satisfactorily close agreement. Attempts to relate the observed variation in enzyme activity to age of patient, sex, total mass of the organ, histologic differences and to the clinical syndrome failed. This indicated the independence of this oxidative enzyme system from these variables and also the essential stability of this biologic mechanism.

TABLE 2.—*Diseased lymph node, tonsil and buffy coat tested for oxidative activity*

Case	Sex	Age	Tissue	Diagnosis	Oxidative activity	
					Q_{O_2} Hydroquinone substrate	Q_{O_2} Succinate substrate
1	M	39	Lymph node	Hodgkin's disease, fibrotic	0. 54	1. 37
2	M	54	" "	" " granuloma	. 5	1. 0
3	F	46	" "	" " sarcoma	0	. 7
4	M	60	" "	" " paragranuloma	1. 36	
5	M	42	" "	Lymphosarcoma, small lymphocytic	. 51	. 73
6	M	42	" "	" "	. 52	. 83
7	F	60	" "	" "	. 43	1. 24
8	F	50	" "	" "	. 19	. 57
9	F	70	" "	" large cell	. 19	. 49
10	M	45	" "	Lymphoid hyperplasia	. 28	. 71
11	F	66	" "	Metastatic adenocarcinoma	. 55	1. 51
12			Tonsil	Simple hyperplasia	1. 1	
13			Buffy coat	Lymphatic leukemia	. 9	

In two instances, the experiments were performed on splenic tissue removed from two separate areas on the basis of morphologic differences. Thus, in one case of Hodgkin's disease of the spleen, an experiment was performed on the nodular Hodgkin's tissue that was gray and firm (fig. 1), and another experiment on the uninvolved intervening tissue. The experiment was repeated on two different areas of a second spleen (fig. 2), one area nodular (megakaryocytoma, microscopically), and the other, red pulp. In both these experiments the oxidative activities of the two areas differed (text-fig. 2).

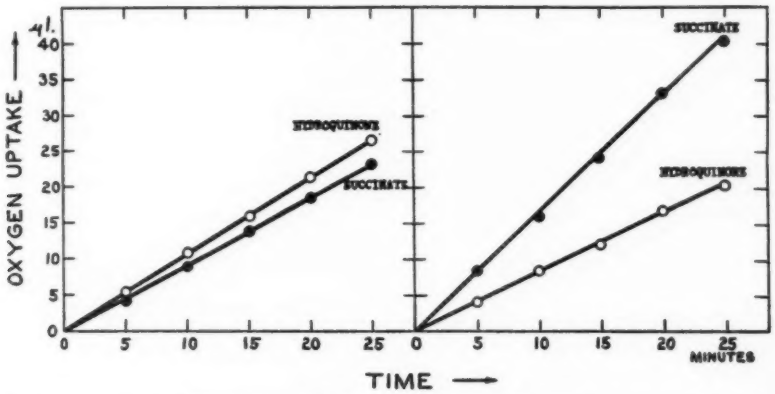
It is noteworthy that the great majority of these specimens manifested appreciably greater activity toward succinate than toward hydroquinone (22 of 26 specimens). This difference is well illustrated in text-figure 1.

It must be emphasized that results such as these depend upon maintenance of a closely-defined set of experimental conditions in contrast to the Q_{O_2} values determined for intact tissue, which are relatively independent of variables such as pH and glucose concentration. Thus, an increase in the *cytochrome c* concentration will produce a corresponding increase in the rate of hydroquinone and succinate oxidation until a saturation level of cytochrome c is reached. As an example, text-figure 3 shows the results of an hydroquinone experiment done with spleen #585, removed from a patient with peptic ulcer, where the cytochrome c concentration was varied from 1.8×10^{-5} M to 11.1×10^{-5} M, with a resultant increase of Q_{O_2} from 0.2 to 2.1.

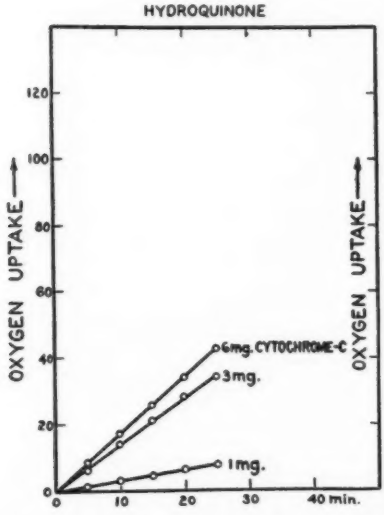
From similar experiments with succinate it appeared that the saturation value for this substrate is in the neighborhood of 22×10^{-5} M cytochrome c concentration, *i. e.*, at a level approximately 10 times that of the standard value employed.

TABLE 3—*Spleens tested for oxidative activity*

Case No.	Age	Sex	Clinical syndrome	Anatomical features pertaining to spleen						Other findings	Oxidative activity	
				Weight	Estimate of lymph cells	Endothelial swelling	Congestion	Pulp hyperplasia	Hematopoiesis		Q_{S_2} Hydroquinone substrate	Q_{S_2} Succinate substrate
					Percent							
2,665	55	F	Hypersplenism	460	10	Present	+	+	+	Cirrhosis of liver	0. 38	1. 12
133	57	M	"	400	20	0	++	+	+	"	. 57	1. 06
196	45	M	Anemia	1, 000	5	Present	+	+++	++	Aneurysm pressing on splenic vein.	. 88	1. 16
1,150	49	M	Hypersplenism	1, 000	5	"	0	+++	++	Cirrhosis of liver	. 19	. 37
2,841	64	F	"	2, 550	10	0	0	++++	++++	See figure 2	1. 00	1. 90
			" (See fig. 2)	2, 550	10	0	0	++	++++		2. 10	3. 60
2,989	71	M	Hypersplenism	1, 350		Present	+	++++	++		. 24	. 52
2,936	30	F	Anemia	1, 075	0	"	++++	0	+		. 36	. 38
1,542	25	M	"	430	5	"	0	0	+	Pressure by pancreatic cyst on splenic vein.	. 29	. 85
148	51	F	Splenomegaly due to tuberculosis.	530	5	"	+	+++	++		. 36	. 45
2,894	59	M	Cancer of stomach	243	10	0	+	0	0	Cancer of stomach	2. 69	3. 78
585	59	M	Peptic ulcer	77	30	Present	+	0	0		. 2	1. 3
1,191	58	F	Lymphosarcoma	240	40	"	0	0	0		. 35	. 91
1,190	30	F	Hodgkin's disease	400	30	"	+	0	0		. 20	. 23
2,937	62	F	" "	600	5	"	+	0	0		. 47	. 88
			" (See fig. 1)		0	A Hodgkin's nodule analyzed					. 62	. 56



TEXT-FIGURE 2.—Oxidative activity in two anatomically different areas of a spleen with Hodgkin's disease (*cf.* fig. 1): a) Hodgkin's node; b) surrounding spleen tissue.



TEXT-FIGURE 3.—Spleen from patient with peptic ulcer. Oxidative activities with hydroquinone and varying amounts of cytochrome c. Spleen normal.

Upon varying the concentration of succinate from 7.1×10^{-2} to 7.1×10^{-4} M while maintaining the cytochrome c concentration at 1.8×10^{-5} M, no change in oxygen uptake of a human spleen homogenate was observed, indicating that the enzyme was saturated with the substrate at a succinate concentration of 7.9×10^{-3} M, being the same concentration as that employed in the other experiments. When, however, the hydroquinone concentration was varied between 1.8×10^{-2} and 7.1×10^{-4} M a slightly higher oxygen uptake was observed with the same tissue at 1.8×10^{-2}, as compared with the standard concentration of

7.9×10^{-3} M. The addition of 1-phenyl semicarbazide, as recommended by Quinlan-Watson (*11*) for the purpose of trapping the benzoquinone formed by the oxidation of hydroquinone, had no effect on the rate of the reaction, probably because of the low levels of oxidase activity here involved. However, a shift in pH from 7.1, as ordinarily employed in these experiments to 7.4, produced a high rate of autoxidation of the hydroquinone, and this serves to emphasize the importance of close control of the experimental conditions under which oxidase assays are performed. The same is true for the control of the temperature, since a rise from 25.2° to 37.0° C. will produce a similar increase in the rate of spontaneous substrate oxidation [*cf.* Haas (*3*)].

Purification of the hydroquinone by vacuum sublimation did not affect the rate of its oxidation by tissue homogenates.

It was found that storage of tissue in the frozen state (at −20° C.) for extended periods of time impairs its oxidase activity toward hydroquinone to a greater degree than its oxidative activity toward succinate. This impairment is being studied further.

DISCUSSION

A major problem encountered in the experiments on human tissue was the difficulty in obtaining normal tissue. We could procure no indisputably normal lymph-node material whatever; the best approximation was to utilize as many nodes as could be obtained from patients with a variety of diseases, neoplastic and otherwise, and trust that significant differences might thus be brought out. When, as in the case of spleen, we tried using the normal spleen of a pig instead of human spleen, we found such a difference in oxidative activity that we were confronted with a new question: was the human spleen that was considered histologically to be normal, although obtained from a patient with a peptic ulcer, biochemically abnormal, or did the divergence of cytochrome oxidase activity represent a species difference? The question remains unanswered.

Regardless of these difficulties, the experiments indicated that the oxidative activity was low for all lymphoid tissue from whatever source,—thymus, lymph nodes or spleen. This was true for these tissues regardless of whether they came from patients with cancer or otherwise. *It was impossible, therefore, to categorize Hodgkin's disease either as cancer or noncancer on the basis of the work reported in this paper.*

From results obtained on tissue homogenates, we were unable to distinguish varying activities referable to difference in cytologic composition of the tissue, except in two instances of spleen where two morphologically different areas from the same organ were examined. This may mean that each source material has an oxidative behaviour peculiar to itself, and that to establish accurately differences in behaviour, both the normal and diseased tissue must come from the selfsame individual or animal and that, failing this prerequisite, significant differences may go undetected.

By the manometric method, we were unable to correlate varying oxidative activities with varying cytologic composition of the tissue, but

histochemical studies of cytochrome oxidase activity as evidenced by the Nadi reaction [Hoffmann, *et al.*, (*4*)] in individual cells from the same source material used in the experiments reported above, showed clearly that the oxidase activity of the reticulum cells was higher than that of the lymphocytes (figs. 3 and 4). On the basis of the Nadi reaction, higher activity for tissues rich in reticulum cells would be expected. However, quantitatively this appeared to be weakly true only in the case of spleen #2,937 where hydroquinone was used as a substrate in the manometric experiments.

The most significant result of these experiments was the observation that in the large majority of tissue homogenates examined the rate of oxidation was higher with succinate than with hydroquinone. This would seem to be at variance with the current view that both substrates react with molecular oxygen through the mediation of the cytochrome oxidase-cytochrome c system. Inasmuch as it is generally believed that hydroquinone is oxidized directly by ferricytochrome c [Keilin and Hartree (*5*)], whereas succinate requires the presence of succinic dehydrogenase and additional factors [Stern and Melnick (*13*) and Straub (*14*)], cellular homogenates would be expected either to exhibit the same activity toward both substrates or a greater activity toward hydroquinone than toward succinate. Possible explanations, with support for each, of the fact that the reverse is true in numerous instances listed in tables 1, 2, and 3 are as follows:

(*a*) Hydroquinone and succinate oxidation proceed *via* two independent enzymatic pathways. Supporting this explanation is the fact that the photochemical spectrum of the succinic oxidase system of heart muscle of the rat [Melnick (*7*)] differs significantly from the photochemical spectrum of the respiratory enzyme in intact avian tissue [Philips (*9*)]; the visual absorption spectrum of a heart muscle oxidase preparation reduced with succinate is different from that obtained with ascorbic or hydroquinone as reductants [Keilin and Hartree (*6*)]. This possibility is further strengthened by the difference in stability toward low temperatures.

(*b*) The utilization of added cytochrome c varies with the nature of the substrate employed. This explanation is favored by the observation that a purified oxidase preparation of heart muscle is able to oxidize *p*-phenylenediamine at a rapid rate without the addition of cytochrome c, whereas other substrates, hydroquinone for example, require the presence of added cytochrome c for their oxidation [Keilin and Hartree (*5*)].

(*c*) Hydroquinone requires for its oxidation by ferricytochrome c a special catalyst or enzyme which is present in insufficient concentrations in the tissues exhibiting the above-mentioned anomaly. Preliminary spectroscopic experiments designed to test the validity of this last possibility have thus far yielded no conclusive results.

SUMMARY AND CONCLUSIONS

A study was made of the terminal oxidase system of lymphoid tissue to see whether criteria for distinguishing neoplastic from nonneoplastic

diseases of the lymphoid system could be established. The material studied consisted of spleens, lymph nodes and thymus glands from patients with a variety of diseases belonging in the above-mentioned categories.

The oxidative activities of homogenates prepared from this material were tested on hydroquinone and succinate as substrates, using the Warburg manometric method.

The experiments disclosed that whether the tissue was neoplastic or nonneoplastic, oxidase activities could not be correlated with any one of the many clinical or pathologic variables, except in two instances when comparisons were made on tissue samples from cytologically different areas of the same organ.

An unexpected finding was that for most of the tissues examined the rate of succinate oxidation was higher than the rate of hydroquinone oxidation. On the basis of current theories either equal activity toward both substrates or a greater activity toward hydroquinone would be expected. The theoretical implications of this finding are discussed.

ADDENDUM

To examine further the apparent anomaly observed in this study that certain tissues show an appreciably greater oxygen uptake with succinate than with hydroquinone as the substrate, the heart muscle of a freshly slaughtered steer was trimmed of fat, washed free of blood with water, ground in a hand meat grinder, and thoroughly mixed by hand. A portion of the tissue was homogenized for an immediate manometric determination of oxidase activity while the remainder was stored in closed containers at +5°, −20°, and −80° C. for varying periods of time. In the *fresh* tissue the enzymatic activity toward both succinate and hydroquinone was essentially the same, namely Q_{O_2} 24.3 (succinate) and 23.8 (hydroquinone), respectively. Manometric experiments performed on aliquots of the same tissue after storage periods of 2, 8, 9, and 17 days at 5° C. showed that the average oxidase activity toward *hydroquinone* as substrate exceeded the average oxidase activity toward succinate as substrate by a ratio of 2.9:1.

The tissue kept frozen at *−20° C.* showed, in experiments performed after 7, 14, 20, 23, 29, and 36 days of storage, a higher oxidase activity toward succinate than toward hydroquinone, the average ratio of cytochrome oxidase (hydroquinone) to succinic oxidase activity being 1:2.

The tissue kept frozen at *−80° C.*, after 3, 10, 14, 20, 24, and 29 days of storage, also displayed a higher succinic oxidase activity, the ratio of cytochrome oxidase (hydroquinone) to succinic oxidase activity being 1:2. The results are summarized in the following table:

History of tissue	Days in storage	Oxidase activity		Ratio $\frac{Q_{O_2}\ (Hq)}{Q_{O_2}\ (S)}$
		Q_{O_2} (*Hq*) Hydroquinone	Q_{O_2} (*S*) Succinate	
Fresh		23.8	24.3	0.98
Kept at +5° C	2	33.6	22.0	1.5
	8	19.2	9.5	2.0
	9	26.1	5.5	4.8
	17	17.3	5.5	3.1
Average ratio				02.9
Kept at −20° C	7	13.0	19.2	.7
	14	8.2	15.2	.5
	20	8.1	24.2	.3
	23	12.0	23.9	.5
	29	8.7	20.2	.4
	36	8.8	19.1	.5
Average ratio				0.5
Kept at −80° C	3	13.7	19.5	.7
	10	15.1	23.9	.6
	14	8.4	21.5	.5
	20	9.9	21.9	.5
	24	10.2	20.1	.5
	29	8.3	17.9	.5
Average ratio				0.6

The differential effect of storage of the heart tissue at different temperatures on the relative activities toward the two substrates employed constitutes further evidence in favor of the assumption that the aerobic oxidation of hydroquinone and succinate proceeds *via* separate enzymatic pathways.

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PLATE 90

FIGURE 1.—Spleen from Hodgkin's disease. Oxidative activity of area "a" differed from that of area "b": (a) nodular gray Hodgkin's tissue; (b) intervening uninvolved tissue.

FIGURE 2.—Spleen from a case of hypersplenism with splenomegaly. Oxidative activity of area "a" (megakaryocytoma) differed from that of area "b" (intervening splenic pulp).

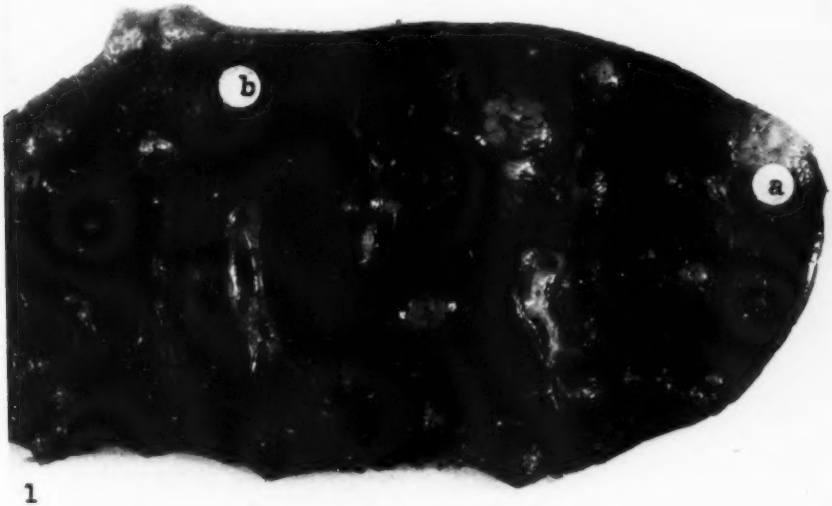

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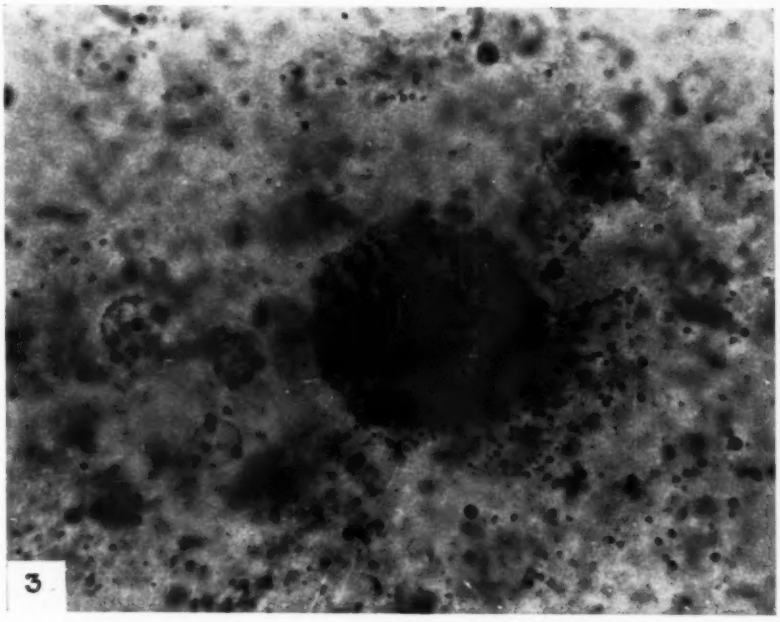
PLATE 91

FIGURE 3.—Nadi reaction within a reticulum cell. Each granule indicates a locus of oxidative activity.

FIGURE 4.—Nadi reaction within lymphocytes.



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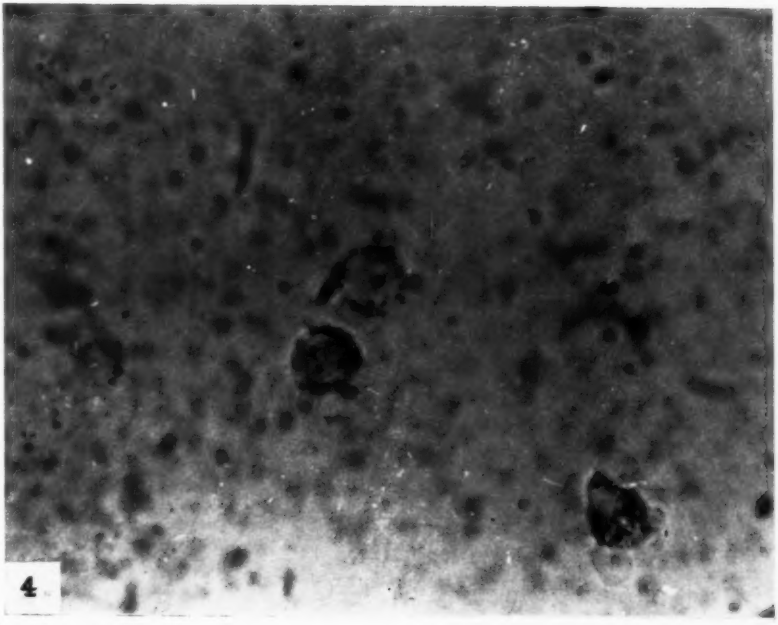

4

STUDIES ON THE BIOLOGICAL INTERRELATIONSHIPS OF FOLIC ACID, CITROVORUM FACTOR, AND THE ANTIMETABOLITE, AMINOPTERIN [1] [2]

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The administration of folic acid (FA) has been only partially successful in preventing the systemic toxicity of repeated injections of the antimetabolite 4-amino-pteroylglutamic acid (aminopterin) in the mouse, rat, and chick (*1-6*). We have recently demonstrated that the toxicity of individual supralethal doses of aminopterin for mice is blocked successfully by relatively low doses of FA when the drugs were administered repeatedly at not less than weekly intervals (*2*). Citrovorum factor (CF), in contrast, was capable of preventing the development of toxicity of supralethal doses of aminopterin even upon daily administration of metabolite and antagonist (*3*).

Citrovorum factor, first described by Sauberlich and Baumann (*7*), as an essential growth factor for Leuconostoc citrovorum, is a biologically active derivative of pteroylglutamic acid (FA) (*8-15*). Brockman *et al.* (*16*) prepared a crystalline substance (Leucovorin),[6] by synthesis from FA, and Flynn *et al.* (*17*) obtained a synthetic factor (Folinic acid-SF) from FA with biological properties identical with CF (*16, 18*). A structure for Folinic acid-SF has been proposed (*19*).[7]

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[2] The authors wish to acknowledge the technical assistance of Annie Collins, Natalie Hill, Vera Rhein, and Audrey O'Connell. The authors are indebted to Nathan Mantel and Samuel Greenhouse of the Biometrics Section of the National Cancer Institute for their helpful suggestions and invaluable aid with the statistical analyses. These investigations were aided in part by a grant from the American Cancer Society to the Department of Preventive Medicine, The Johns Hopkins University School of Medicine, recommended by the Committee on Growth of the National Research Council.

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[6] Leucovorin, designated in this paper as CF. Leucovorin (CF), folic acid (FA), and aminopterin were kindly provided by the Lederle Laboratories Division of the American Cyanamid Co. The Leucovorin (CF) contained 20 million units per ml., equivalent to 3 mg. CF per ml.

[7] Leucovorin and Folinic acid-SF may be identical (*19*).

On the basis of *in vitro* and *in vivo* studies, Nichol and Welch (*13, 20*) suggested that aminopterin acts by competitive saturation of the enzymes involved in the conversion of FA to CF and in the further utilization of CF. Broquist *et al.* (*11*) demonstrated that CF reversed the inhibitory effect of aminopterin in a competitive manner in bacteria and mice. Attention was drawn to a probable role of CF in the catalytic formation of thymidine from desoxyribosides (*12*), a reaction which may be blocked by aminopterin (*11*). Other studies (*2, 3, 21*) supported the hypothesis that aminopterin blocks enzymatic conversion of FA to CF, as well as enzymatic activity involved in the further metabolism of CF. Animals protected from aminopterin by either CF or FA possessed "masked enzymatic deficiencies," which became apparent upon subsequent administration of the metabolites with the antagonist.

The present experiments were undertaken to investigate further *a*) the temporal relationships of FA and CF administration to aminopterin toxicity; *b*) the competitive relationships of FA and of CF to aminopterin; and *c*) the inhibitory role of aminopterin in the conversion of FA to CF and on the further utilization of CF.

METHODS

In previous studies (*2,3*), a fixed lethal dose of aminopterin (10 mg./kg.) was employed, together with CF or FA. In the present studies, the median lethal dose (LD_{50}) for aminopterin was usually employed, in order to obtain a more precise expression of the interrelationships of FA and CF to aminopterin toxicity. In a typical experiment, the mortality was determined at a series of dose levels of aminopterin with a particular dosage of FA or CF. The LD_{50} and 19/20 confidence limits were estimated according to the method of Litchfield and Wilcoxon (*22*). The method was employed to determine the relative activity of aminopterin in the presence or absence of FA or CF. In computation of the relative potency, it was first determined that the slopes did not deviate significantly from parallelism.

The experiments were conducted with strain A and strain CFW male mice (NCI) weighing 18 to 22 grams.[8] The mice were housed in plastic cages and received Purina laboratory chow and water *ad libitum*. They were maintained at 72° to 78° F. prior to, and during the course of, the experiments. All injections were subcutaneous into the axillary space. When more than one agent was administered, the agents were injected into opposite axillae. The mice were observed daily for mortality for a period of 10 days subsequent to the last day of administration of aminopterin. The dosage schedules are indicated with the individual experiments. The CF was diluted in 0.5 percent sodium bicarbonate. The FA and aminopterin were administered in 2 percent sodium bicarbonate. The total volume of solution for each drug administered usually did not exceed 1 percent of the body weight of the mice.

[8] No significant differences were observed in the dose-response relationships with these two strains of mice.

RESULTS

The experimental data are summarized in tables 1 through 4 and graphically represented in text-figures 1 through 7.

TABLE 1.—*Effect of delayed administration of citrovorum factor on the lethal toxicity of aminopterin single and multiple treatment with citrovorum factor*

Dose (mg./kg.)		Time of administration of citrovorum factor in relation to aminopterin (hours)	Mortality
Aminopterin	Citrovorum factor		
20	30	+24	6/6
10	30	+24	1/6
5	30	+24	1/6
20	7.5	+24, 48, 72, 96	6/6
10	7.5	+24, 48, 72, 96	2/6
5	7.5	+24, 48, 72, 96	3/6
20			6/6
10			6/6
5			5/6

CFW and A male mice. All injections subcutaneous. Aminopterin and CF administered simultaneously.

TABLE 2.—*Effect of pretreatment with citrovorum factor plus aminopterin on the subsequent protection afforded by folic acid against aminopterin toxicity*

	Day 0 (dose mg./kg.)		Day 1 (dose mg./kg.)		LD_{50} (19/20 confidence limits). Mg./kg. aminopterin
	Citrovorum factor	Aminopterin	Folic acid	Aminopterin	
I	7.5	10		0.06–10.0	0.70 (0.32–1.50)
II	7.5	10	25	.06–10.0	.52 (.29–.94)
III	7.5			1.0 –10.0	6.00 (3.73–9.66)

LD_{50} are calculated on the basis of treatment day 1. Citrovorum factor and aminopterin were administered simultaneously. Folic acid was administered 1 hour prior to aminopterin. Number of mice employed: Group I—100; group II—100; group III—60. CFW and A male mice. All injections subcutaneous.

TABLE 3.—*Effect of pretreatment with folic acid plus aminopterin and citrovorum factor plus aminopterin on the subsequent protection afforded by citrovorum factor against aminopterin toxicity*

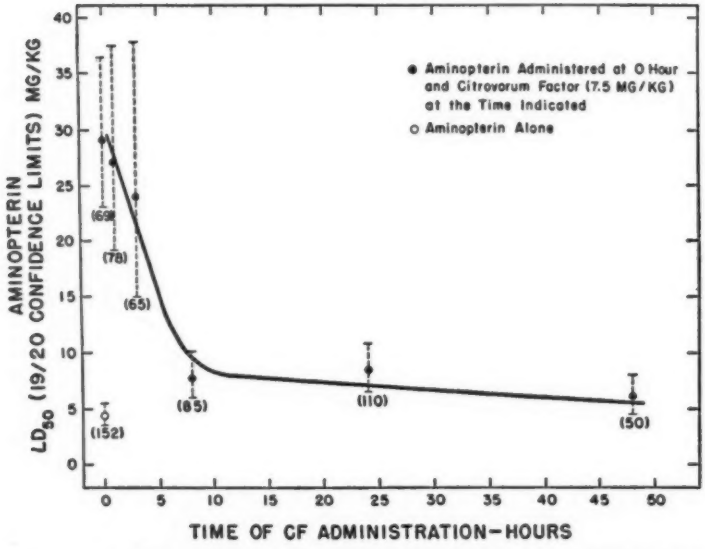
	Day 0 (dose mg./kg.)			Day 1 (dose mg./km.)		LD_{50} (19/20 confidence limits). Mg./kg. aminopterin
	Folic acid	Citrovorum factor	Aminopterin	Citrovorum factor	Aminopterin	
I	25		10		(0.063– 1.0)	0.70 (0.46– 1.06)
II	25		10	7.5	(5.0 –40.0)	15.81 (10.97–22.75)
III		7.5	10		(.063– 2.0)	.64 (.38– 1.09)
IV		7.5	10	7.5	(5.0 –40.0)	16.1 (10.0 –25.92)
V					(1.0 –10.0)	5.20 (3.94– 6.86)
VI				7.5	(5.0 –40.0)	29.5 (22.52–38.65)

The LD_{50} are calculated on the basis of treatment day 1. Citrovorum factor and aminopterin were administered simultaneously. Folic acid was administered 1 hour prior to aminopterin. 40–72 mice per group. CFW and A male mice. All injections subcutaneous.

TABLE 4.—*Effect of citrovorum factor on the toxicity of multiple daily doses of aminopterin*

Daily dose (mg./kg.)		Number of days of treatment	Mortality (dead/total)
Citrovorum factor	Aminopterin		
7.5	10	8	7/7
7.5	5	11	2/7
7.5	2.5	11	0/7
	2.5	4	7/7

CFW and A male mice. All injections subcutaneous. CF and aminopterin administered simultaneously.

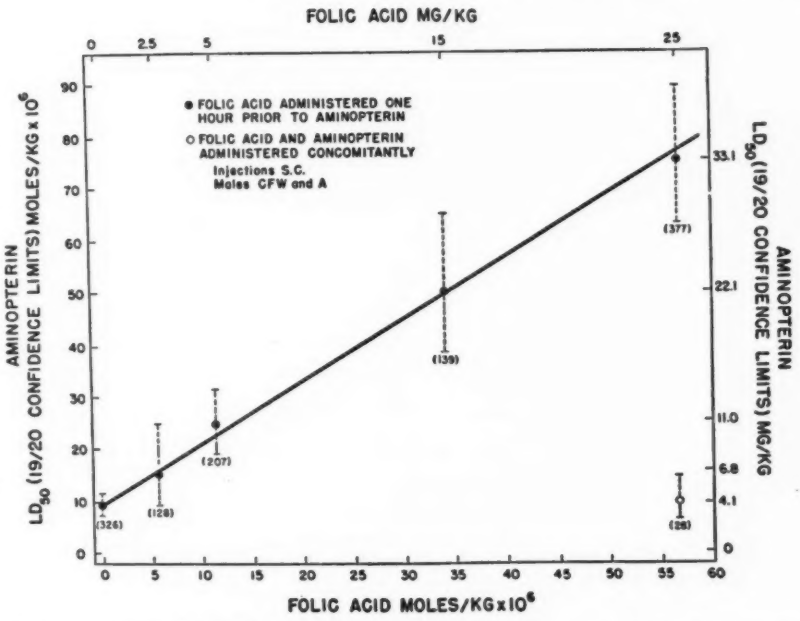


TEXT-FIGURE 1.—The relationship of the time of administration of citrovorum factor to the protection afforded against aminopterin toxicity. CFW and A male mice. All injections were given subcutaneously. Number of animals in parentheses.

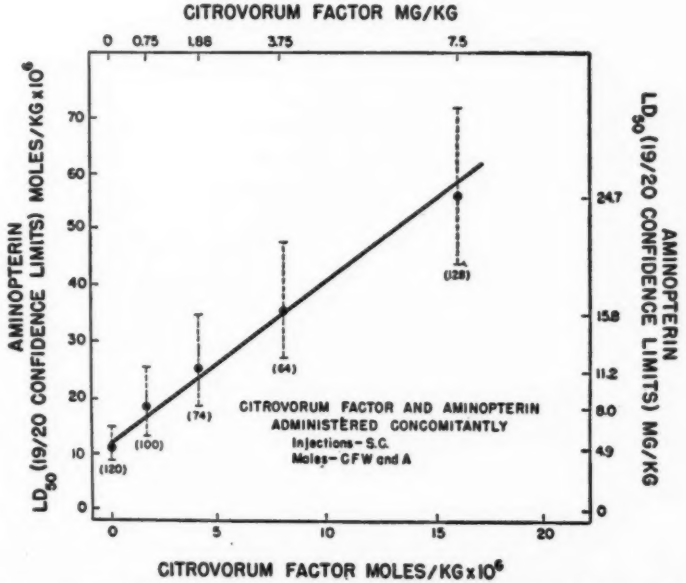
TEMPORAL RELATIONSHIPS

FA afforded a significant increase in protection against aminopterin toxicity when administered 1 hour prior to aminopterin (text-fig. 2). The ratio (R. LD_{50})[9] and its 19/20 confidence limits of the median lethal dose of aminopterin, with added FA (25 mg./kg. FA administered 1 hour early), as compared with that of aminopterin alone, was 8.1 (6.1 to 10.7) (text-fig. 2). In contrast, no protection against aminopterin toxicity was provided by a dose of 25 mg./kg. of FA when the FA was administered simultaneously with the aminopterin (text-fig. 2), or 24 hours prior to aminopterin (text-fig. 6). Administration of 25 mg./kg. FA 24 hours prior, followed by administration of 25 mg./kg. FA 1 hour prior to aminopterin, resulted

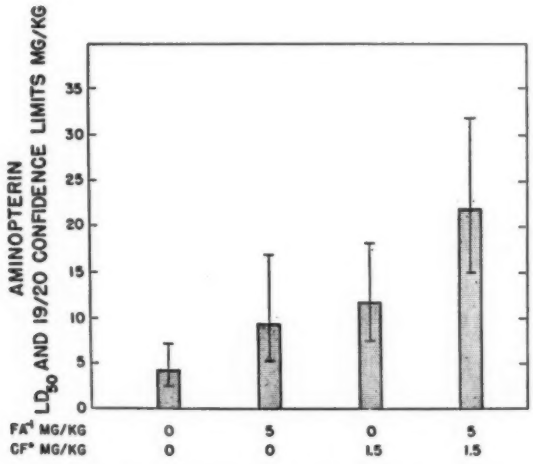
[9] R. LD_{50} and 19/20 confidence limits are equivalent to the potency ratio of the LD_{50} of Litchfield and Wilcoxon (22). Confidence limits of the R. LD_{50} are in parentheses.



TEXT-FIGURE 2.—Effect of folic acid on aminopterin toxicity in mice. Number of animals in parentheses.



TEXT-FIGURE 3.—Effect of citrovorum factor on aminopterin toxicity in mice. Number of animals in parentheses.

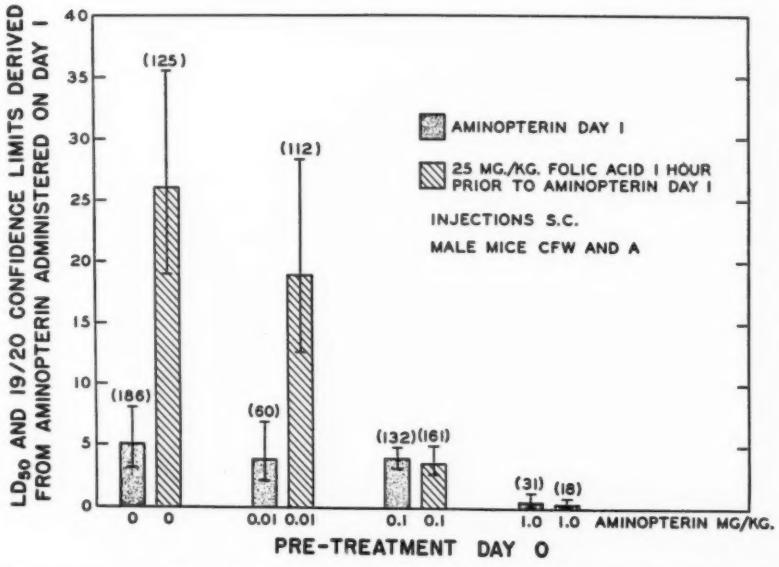


TEXT-FIGURE 4.—Effect of administration of folic acid plus citrovorum factor on aminopterin toxicity. CFW and A male mice. All injections were given subcutaneously. FA^{-1}=folic acid administered 1 hour prior to aminopterin. CF^{0}= citrovorum factor administered concomitantly with aminopterin. Sixty-four animals per group.

in no further increase in protection (text-fig. 6) when compared with the results obtained on administration of a single dose of FA 1 hour prior to the aminopterin (text-fig. 2).

In contrast with FA, CF provided significant protection against aminopterin toxicity when the CF and aminopterin were administered simultaneously (text-figs. 1, 3, 7). The R. LD_{50s} of aminopterin plus 7.5 mg./kg. CF administered simultaneously, as compared with aminopterin alone, were 6.6 (4.8 to 9.0); 5.0 (3.5 to 7.3); 6.4 (4.2 to 9.9) (text-figs. 1, 3, 7). Administration of 7.5 mg./kg. of CF 1 hour or 3 hours subsequent to the aminopterin, likewise resulted in significant increase in protection as compared with aminopterin alone [R. LD_{50s}=6.1 (4.2 to 9.1) and 5.4 (3.3 to 9.1)] (text-fig. 1). However, there was a marked decline in the degree of protection afforded by 7.5 mg./kg. CF when it was administered at 8 or 24 hours subsequent to the aminopterin (text-fig. 1). The R. LD_{50s} as compared with aminopterin alone were: at 8 hours, 1.8 (1.2 to 2.5); at 24 hours, 1.9 (1.4 to 2.8). With administration of CF (7.5 mg./kg.) 48 hours subsequent to aminopterin, the difference noted as compared with aminopterin alone was not significant. A tenfold increase in the dose of CF (75 mg./kg.) administered 24 hours after the administration of aminopterin resulted in a significant increase in the protective action of the CF [LD_{50}= 17.0 (9.4 to 30.8)],[10] as compared with the result obtained on administration of 7.5 mg./kg. CF administered at the same time interval [R. LD_{50s}= 2.0 (1.04 to 3.8)]. However, when the same dose of CF (75 mg./kg.) was administered 48 hours subsequent to the aminopterin, no significant

[10] Confidence limits of LD_{50} in parentheses.



TEXT-FIGURE 5.—The effect of pretreatment with aminopterin on the subsequent protection afforded by folic acid against aminopterin toxicity. Twenty-four hours after each "pretreatment" dose of aminopterin (indicated at the base of chart), 1 group of animals was injected at a series of dose levels of aminopterin alone, while the other group received 25 mg./kg. of folic acid 1 hour prior to aminopterin, plus a series of dose levels of aminopterin. The LD_{50} (mg./kg.) were calculated from the observed mortality at the dose levels of aminopterin employed in treatment day 1.

Calculated as the treatment dose following which 50 percent of the animals died. Alternatively, the LD_{50} could have been calculated as the treatment dose following which the percentage mortality equals the pretreatment percentage mortality plus half the pretreatment percentage survivorship. This calculation, however, fails to reflect the extent of reversibility of pretreatment dosage damage, and in any case would not alter the conclusions reached here. Number of animals in parentheses.

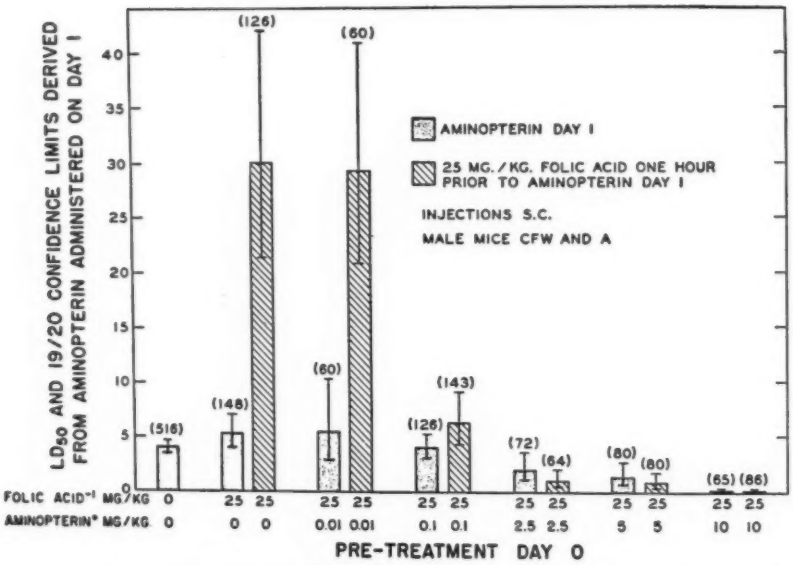
increase in protection was noted. Administration of 7.5 mg./kg. CF 24 hours prior to aminopterin did not result in a significant increase in protection [LD_{50}=6.0 (3.7 to 9.7)], as compared with the result observed after treatment with aminopterin alone [11] [R. LD_{50}=1.4 (0.8 to 2.3)].

When administration of CF was delayed, repeated divided doses were somewhat less effective than a single dose in preventing aminopterin toxicity (table 1).

THE COMPETITIVE RELATIONSHIPS OF FA AND CF TO THE TOXICITY OF AMINOPTERIN

Since optimal protection against aminopterin toxicity appeared to be afforded by administration of FA 1 hour prior to aminopterin (*2*), the effect of FA on aminopterin toxicity was studied in more detail employing this temporal relationship of metabolite and antagonist. The LD_{50} for aminopterin was determined at a series of dosage levels of added FA

[11] See text-figure 1.



TEXT-FIGURE 6.—The effect of pretreatment with folic acid and aminopterin on the subsequent protection afforded by folic acid against aminopterin toxicity. Twenty-four hours after each "pretreatment" dose combination of folic acid and aminopterin, 1 group was injected at a series of dose levels of aminopterin alone, while the other group received 25 mg./kg. of folic acid 1 hour prior to aminopterin, at a series of dose levels of aminopterin. The LD_{50} (mg./kg.) were calculated from the observed mortality at the dose levels of aminopterin employed in treatment day 1. Folic acid^{-1}= folic acid administered 1 hour prior to aminopterin (Aminopterin [9]). Number of animals in parentheses.

ranging from 0 to 25 mg./kg. of FA. The latter dose was chosen as the upper limit since at doses greater than 25 mg./kg. the toxicity of FA itself may become manifest (*2*), and preclude accurate assay. A linear relationship was evident between the median lethal dose of aminopterin and FA administered 1 hour prior to aminopterin (text-fig. 2). The equation describing the linear relationship is $I=1.19$ (0.99 to 1.39)$S + 9.46 \times 10^{-6}$ (7.43×10^{-6} to 11.49×10^{-6}).[12]

A linear relationship between the median lethal dose of aminopterin and added CF was likewise apparent when the CF was administered concomitantly with the aminopterin (text-fig. 3). The equation describing the relationship is $I = 2.98$ (2.24 to 3.72)$S + 11.69 \times 10^{-6}$ (9.04×10^{-6} to 14.34×10^{-6}) (text-fig. 3).[13]

The linear relationships in text-figures 2 and 3, are described by the equation $I=mS+b$, in which m represents the slope of the respective lines and b is equal to the LD_{50} of aminopterin alone. If it is assumed that the lines may be extrapolated through the abscissa, the equation relating the

[12] I=Aminopterin LD_{50} moles/kg.; S=Substrate added (FA moles/kg.). The figures in parentheses represent 19/20 confidence limits.

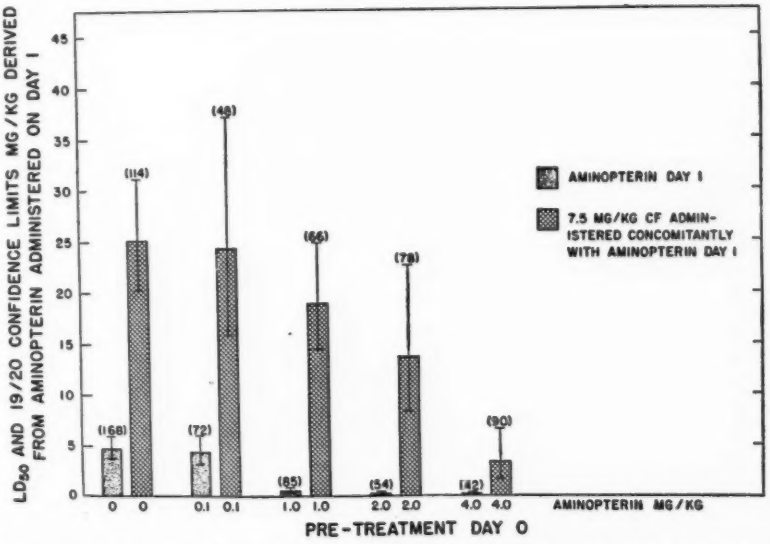
[13] I=Aminopterin LD_{50} moles/kg.; S=Substrate added (CF moles/kg.).

LD_{50} of aminopterin to added FA or CF may be expressed in the form I= m (S + C) in which C represents the endogenous reserve of protection present in the normal mouse. C may be calculated from this equation and may be expressed as FA administered 1 hour prior to, or as CF administered concomitantly with, the aminopterin. For the line relating LD_{50} aminopterin to FA (text-fig. 2) $C=7.95 \times 10^{-6}$ moles/kg. FA. For the line relating LD_{50} aminopterin to CF (text-fig. 3) $C=3.92 \times 10^{-6}$ moles/kg. CF.

The ratio of the slopes (text-figs. 2, 3) is a reflection of the relative protection afforded by CF as compared with FA. The slope ratio is 2.5, indicating that CF administered concomitantly with aminopterin is 2.5 times as effective as FA administered 1 hour prior to aminopterin, in affording protection against aminopterin toxicity.

Cumulative Protection by FA plus CF

The protection afforded by a single dose of FA administered 1 hour prior to aminopterin followed by a single dose of CF administered concomitantly with the aminopterin was cumulative (text-fig. 4). The R. LD_{50}s as compared with aminopterin alone, are 2.2 (1.0 to 4.8) for FA plus aminopterin; 2.8 (1.4 to 5.4) for CF plus aminopterin, and 5.1 (2.7 to 9.8) for FA and CF plus aminopterin.



Text-figure 7.—The effect of "pretreatment" with aminopterin on the subsequent protection afforded by citrovorum factor against aminopterin toxicity. Twenty-four hours after each "pretreatment" dose of aminopterin one group was injected at a series of dose levels of aminopterin alone, while the other group received 7.5 mg./kg. citrovorum factor concomitantly with aminopterin, at a series of dose levels of aminopterin. The LD_{50}s were calculated from the observed mortality at the dose levels of aminopterin employed in treatment day 1. CFW and A male mice. All injections were given subcutaneously. Number of animals in parentheses.

MULTIPLE DOSE TOXICITY

Extent of Protection Afforded By FA After Pretreatment With a Single Sub-LD_{50} or Nontoxic Dose of Aminopterin

Preliminary experiments had indicated that aminopterin was strikingly more toxic when administered on 2 or more consecutive days, than when administered on 1 day. In addition, there was a marked decline in the degree of protection afforded by FA on repeated administration of the drugs, even when the FA was administered 1 hour prior to aminopterin at each injection period.

These experiments were conducted in order to determine how low a dose of aminopterin, administered as pretreatment on day 0, prevented subsequent protection by FA against aminopterin toxicity on day 1. The results observed show (text-fig. 5) that after pretreatment with a dose of 1.0 mg./kg. or 0.1 mg./kg. of aminopterin on day 0 the LD_{50} of the group treated with FA plus aminopterin on day 1 did not differ significantly from the group receiving aminopterin alone on day 1. Pretreatment with these doses of aminopterin apparently blocked subsequent protection by FA. Although the nontoxic dose (0.1 mg./kg.), which is in the range usually employed in tumor studies (1/40 to 1/50 of the single LD_{50} dose), blocked subsequent protection by FA, it did not contribute measureably to the toxicity of aminopterin alone given on day 1. After pretreatment with 0.01 mg./kg. of aminopterin utilization of FA apparently occurred, since the group receiving FA plus aminopterin on day 1 showed a significant increase in the LD_{50}. This dose (0.01 mg./kg.) represents approximately 1/400 to 1/500 of the single dose LD_{50} of aminopterin.

Extent of Protection Afforded By FA After Pretreatment With FA Plus Aminopterin

In these experiments the mice were pretreated with 25 mg./kg. of FA 1 hour prior to aminopterin on day 0, instead of with aminopterin alone (text–fig. 6). No significant increases in the LD_{50} were observed in the groups receiving FA plus aminopterin on day 1, as compared with the groups receiving aminopterin alone on day 1, after pretreatment with FA plus doses of aminopterin ranging from 0.1 to 10 mg./kg. Significant protection by FA on day 1 was observed only in mice receiving FA plus 0.01 mg./kg. aminopterin on day 0. This was comparable in extent to the controls that received FA alone on day 0, and FA plus aminopterin on day 1. Thus, subsequent protection by FA against aminopterin occurred after the same pretreatment dose of aminopterin, regardless of whether FA was administered on day 0.

The Effect of Pretreatment With Aminopterin on the Protection Afforded Subsequently By Citrovorum Factor Against Additional Treatment With Aminopterin.

At pretreatment doses of aminopterin, ranging from 0.1 mg./kg. to 4 mg./kg., the LD_{50} was significantly higher in the groups receiving CF

plus aminopterin on day 1, as contrasted with the corresponding group receiving aminopterin alone on day 1 (text-fig. 7). A pretreatment dose of 0.1 mg./kg. aminopterin did not appreciably alter the ability of CF to protect against subsequent treatment with aminopterin as compared with controls treated with CF plus aminopterin for a single day. This is in contrast to the results with FA (text-fig. 5) in which a pretreatment dose of 0.1 mg./kg. aminopterin prevented subsequent protection by FA. Pretreatment with 1 to 4 mg./kg. aminopterin resulted in a progressive decline in LD_{50} for animals subsequently treated with CF plus aminopterin. Pretreatment with doses of aminopterin ranging from 1.0 to 4.0 mg./kg. resulted in a similar, but proportionately greater, decline in the LD_{50} for animals treated with aminopterin alone on day 1.

Failure of FA to Afford Any Protection Following CF Plus Aminopterin

Following pretreatment with CF plus aminopterin, the LD_{50}s of the groups receiving aminopterin alone, and FA plus aminopterin, did not differ significantly (table 2). Employment of CF with aminopterin in pretreatment failed to permit subsequent protection by FA.

Protective Action of CF After Pretreatment With FA or CF Plus Aminopterin

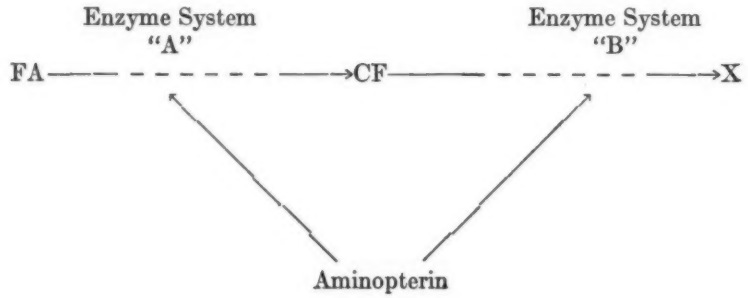
These experiments were conducted to determine whether pretreatment doses of either FA or CF plus aminopterin resulted in any loss of protection by CF against a second dose of aminopterin. After pretreatment with either FA or CF plus aminopterin, the LD_{50} was significantly higher in the group receiving CF plus aminopterin on day 1 (table 3), as contrasted with the corresponding pretreated group receiving aminopterin alone on day 1. The pretreated groups subsequently treated with aminopterin alone showed a sharp fall in LD_{50}, as contrasted with the control group receiving a single dose of aminopterin alone. The pretreated groups subsequently treated with CF plus aminopterin showed significant, but proportionately less, reduction in LD_{50} when compared with the control receiving CF plus aminopterin. These results suggest that the pretreatment dose combinations, although relatively nontoxic *per se*, produce a subsequently measurable degree of physiological injury to the animal.

Effect of Prolonged Treatment With CF Plus Aminopterin

Animals subjected to continuous daily treatment with 7.5 mg/kg. CF plus 10 mg./kg. aminopterin succumbed (table 4). All animals survived when the daily dose of aminopterin was reduced to 2.5 mg./kg.

DISCUSSION

The metabolic transformations involving FA and CF, and the relationship of aminopterin thereto, may be represented by the following schema.[14]



The following observations provide evidence that folic acid acts as a precursor of CF in the mouse. (*a*) Although FA acted competitively when administered 1 hour prior to aminopterin, it afforded no protection against aminopterin toxicity on simultaneous administration. CF not only afforded protection when administered 1 hour prior to aminopterin (*3*), but also acted in a competitive manner when administered simultaneously with aminopterin (text-figs. 2–3). (*b*) A dose of 25 mg./kg. FA, or 7.5 mg./kg. CF, administered 24 hours earlier than aminopterin failed to afford significant protection. Apparently, the rates of utilization and/or excretion of the metabolites are similar (*3*). (*c*) Interference with the utilization of FA resulting from pretreatment with aminopterin (text-fig. 5) did not prevent further utilization of CF (text-fig. 7). (*d*) Cumulative protection was afforded when FA was administered 1 hour prior to aminopterin and CF was administered simultaneously with aminopterin (text-fig. 4).

The data suggest that aminopterin acts as an essentially irreversible inhibitor for the enzyme system "A" involved in the conversion of FA to CF. The 1-hour interval which permits protection by FA against aminopterin toxicity appears to be the time during which metabolic conversion of FA to CF occurs with resultant competitive action of CF for Enzyme "B," rather than the time required for FA to compete successfully for Enzyme "A." These conclusions are supported by the following evidence: (a) Pretreatment with a single, nontoxic dose of aminopterin (0.1 mg./kg.) blocked completely the subsequent utilization of FA (text-fig. 5). When 25 mg./kg. FA was administered 1 hour prior to this low dose of aminopterin (0.1 mg./kg.), no increase in the subsequent utilization of FA was observed (text-fig. 6). Since added FA afforded no protection on simultaneous administration with aminopterin (text-fig. 2), it would appear that the binding of Enzyme "A" not only is irreversible, but is extremely rapid.

[14] The broken lines indicate that the action of aminopterin may be on enzymatic processes involved in transformations of metabolic products derived from FA or CF. The synthetic compound "Leucovorin" may not be the citrovorum factor as derived from liver (*23*).

(b) If the FA protected primarily by successful competition for Enzyme "A," Enzyme "B" would be sensitive, since a low level of CF would obtain at the time of administration of aminopterin. However, this is improbable, since CF afforded protection after pretreatment with a single sublethal dose of aminopterin (text-fig. 7), or after pretreatment with FA administered 1 hour prior to a supralethal dose of aminopterin (table 3). That FA does not act by successful competition for Enzyme "A" is also illustrated by an experiment in which on day 0, FA (12.5 mg./kg.) was administered 1 hour prior to, as well as concomitantly with, aminopterin (5 mg./kg.). Twenty-four hours later, the animals received FA (25 mg./kg.) 1 hour prior to aminopterin (10 mg./kg.). No animals survived, indicating that there was no utilization of FA on the second day of treatment. Apparently neither of the pretreatment doses of FA afforded any protection to Enzyme "A." [15] The observation that pretreatment with CF plus aminopterin did not permit further utilization of FA (table 2. suggests that the process of conversion of FA to CF is nonreversible) Thus, FA, by acting as a presursor of CF, when administered 1 hour prior to aminopterin, gives the appearance of preventing the toxicity of aminopterin in a competitive manner.

A total inhibition of enzyme system "A" as a result of the administration of a single low dose of aminopterin does not appear to contribute to the subsequent toxicity of aminopterin, *i. e.*, a pretreatment dose of 0.1 mg./kg. of aminopterin blocked the subsequent utilization of FA (text-fig. 5), but did not significantly reduce the median lethal dose of aminopterin (text-figs. 5, 7). Apparently, after this treatment, there is a sufficient reserve of CF (or alternate pathways of synthesis of CF) in the animal to tide it over until there has been sufficient restoration of Enzyme "A" for the animal to utilize FA again (*2*). Whether a more prolonged depletion of enzyme system "A" *per se* would ultimately contribute to toxicity cannot be determined from these data.

The inhibition of enzyme system "B" by aminopterin is apparently not strictly competitive,[16] even though a linear relationship was observed between the LD_{50} of aminopterin and the dose of CF, on simultaneous administration of the drugs. The observations indicate that aminopterin is a slowly acting, irreversible inhibitor of enzyme system "B," and that CF interferes with the reaction of aminopterin with Enzyme "B." This action would be comparable, for example, to the interference of succinate with the reaction between copper and succinic dehydrogenase. This type of competition has been referred to as "interference" (*24*). The following observations provide evidence that the aminopterin may bind enzyme system "B." (*a*) A progressive decline in the LD_{50} of aminopterin was observed when the administration of a fixed dose of CF (7.5 mg./kg.) was withheld for increasing intervals after the administration of aminopterin

[15] Unpublished data: Goldin, A., Venditti, J. M., Greenspan, E. M., and Schoenbach, E. B.

[16] All of the essential enzyme systems that may be involved in further metabolic transformations of CF are included in the expression "enzyme system 'B'". It may be that the enzyme system most immediately involved in CF conversion competes reversibly with aminopterin, and that the irreversible alterations are limited to an enzyme system(s) involved in subsequent transformations.

(text-fig. 1). (*b*) A tenfold increase in the dose of CF administered 24 hours after aminopterin did not result in as extensive a rise in the LD_{50} of aminopterin as would have been expected [17] if there had been no inactivation of enzyme system "B." (*c*) Pretreatment with progressively larger doses of aminopterin resulted in a decline in the protection afforded by CF, 24 hours later, when administered with a second dose of aminopterin (text-fig. 7). (*d*) Pretreatment with relatively nontoxic dose combinations of either FA administered 1 hour prior to aminopterin or CF administered simultaneously with aminopterin, followed in 24 hours by CF at a series of dosages of aminopterin, resulted in significant decreases in the LD_{50} (table 3). (*e*) Eleven daily doses of 7.5 mg./kg. of CF plus 2.5 mg./kg. of aminopterin, administered simultaneously, resulted in no deaths. However, all of the animals succumbed after 8 daily doses of 7.5 mg./kg. of CF plus 10 mg./kg. of aminopterin (table 4). (*f*) Repeated delayed administration of CF was not more effective than a single delayed dose of CF in preventing aminopterin toxicity (table 1). This observation suggests that the action of aminopterin is not restricted to a depletion of some substrate replaceable by CF.

However, the toxic action of aminopterin may be partially overcome by CF when the CF is administered 24 hours subsequent to the aminopterin (text-fig. 1). This may also be inferred from the data in text-figure 7. After pretreatment with aminopterin, mice treated with aminopterin plus CF showed proportionately less diminution in LD_{50} than mice subsequently treated with aminopterin alone. In the mice subsequently treated with CF plus aminopterin, it would appear that the CF partially reversed the effect of the pretreatment dose of aminopterin, in addition to affording protection against the second dose of aminopterin. A similar result was noted after pretreatment with FA or CF, plus aminopterin (table 3).

SUMMARY

Toxicity studies were conducted in mice, using the metabolites folic acid (FA) and citrovorum factor (CF), and the antagonist, aminopterin.

A linear relationship was observed between the median lethal dose (LD_{50}) of aminopterin and the dose of CF employed, on simultaneous administration of the drugs. A similar relationship was evident on administration of FA 1 hour prior to aminopterin, but did not obtain on simultaneous administration of the drugs.

There was a progressive decline in the LD_{50} of aminopterin as the administration of CF was withheld for longer intervals of time. A tenfold increase in the dose of CF resulted in only a partial increase in LD_{50}, when withheld for 24 hours, and was without effect when withheld for 48 hours.

Administration of FA 1 hour prior to aminopterin plus CF simultaneously with aminopterin, resulted in an increase in LD_{50} beyond that observed with comparable doses of FA or CF.

[17] By extrapolation from curve (text-fig. 3).

After pretreatment with sub-LD_{50} doses of either aminopterin, FA plus aminopterin, or CF plus aminopterin, treatment with FA administered 1 hour prior to aminopterin did not result in a significant increase in LD_{50}, as compared with corresponding pretreated groups subsequently treated with aminopterin alone. Following similar pretreatments, treatment with CF plus aminopterin resulted in significant increases in LD_{50}. However, the resultant LD_{50}s were diminished as compared with that observed on a single day's administration of CF plus aminopterin.

A single dose of CF was more effective than repeated smaller doses of CF when treatment was withheld for 24 hours following the administration of aminopterin.

Mice tolerated 11 days of treatment with 7.5 mg./kg. CF and 2.5 mg./kg. aminopterin administered concomitantly.

Administration of 7.5 mg./kg. of CF or 25 mg./kg. FA 24 hours prior to aminopterin resulted in no increase in the LD_{50} of aminopterin.

These results are discussed with relation to the mechanism of action of aminopterin on the metabolic transformations involving FA and CF.

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THE TRANSPLACENTAL EFFECT OF URETHAN ON LUNG TUMORIGENESIS IN MICE [1] [2]

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Since the initial observation of Nettleship and Henshaw (*1*) that administration of urethan to mice significantly increased the incidence and decreased the latent period in lung tumorigenesis, the same results have been obtained repeatedly by other investigators with mice (*2*, *7*) as well as rats (*8*, *9*). It also has been observed that lung tumors may be induced in mice with one intraperitoneal injection of urethan (*2*) and that they arise whether the carcinogen is administered by subcutaneous injection or in the drinking water (*3*), by nasal instillation (*7*) or following skin painting (*10*). Larsen (*11*) injected pregnant strain A mice intraperitoneally with urethan at varying periods up to 19 days following the discovery of a vaginal plug, permitted the mice to litter, and separated the offspring according to the time, in days, between injection and parturition. The mice were autopsied 6 months thereafter, when it was observed that lung tumors had been induced in the offspring as well as in the treated mothers. Significantly more tumors were obtained in the young born 1 day as compared with 2 to 5 days following the injection of a pregnant mouse. Law (*12*) previously injected 1,2,5,6-dibenzanthracene *in utero* into the amniotic fluid of Caracul $\times$ PF_1 hybrid mice, and obtained lung tumors among the surviving offspring at an average age of 200 days. In the experiments of Smith and Rous (13), pregnant strain C mice were injected with urethan from about the 12th to the 18th day of gestation. The litters were assembled and the young autopsied at 3, 10, or 60 to 70 days of age. Lung adenomas were observed in treated mothers and in the young not only of the 10-day and 60- to 70-day groups, but even of the 3-day group. Tumors enlarged rapidly so that some reached a diameter of 0.2 mm. in the 10-day old mice. Smith and Rous (*13*) were unable to demonstrate a difference in lung tumor incidence with time between injection of urethan and parturition. Sinclair (*14*) injected urethan into pregnant mice and reported the development of brain anomalies in the young.

In the present experiments, the transplacental effect of urethan on lung

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tumorigenesis in mice was reinvestigated. In one group, the pregnant mice were injected as late as the 19th day following discovery of a vaginal plug, parturition generally occurring within several hours. A higher incidence and more tumors per lung were observed in offspring of these mice compared with the young of mothers injected 1 or 2 days earlier. In another group, pregnant mice were injected on the 19th day and the young delivered by caesarean after 1 or 5 hours. The incidence and multiplicity of lung tumors were unexpectedly high in both caesarean groups.

MATERIALS AND METHODS

The albinos employed in these experiments were derived from a colony of originally strain A mice in which the agouti gene appeared, indicating a possible outcrossing to another albino strain, probably C (BALB/c). The mice were maintained in plastic cages measuring 27.5 by 17.5 by 12.5 cm., generally 8 to a cage, and had access to a continual supply of Purina laboratory chow and tap water. Young adult males and females were mated and a daily record was kept of vaginal plugs. Pregnant females were injected once intraperitoneally with 0.25 cc. of a 10 percent solution of urethan in distilled water on the morning of the 17th, 18th, or 19th day following the discovery of a plug. Cages containing pregnant mice were examined daily for litters, except on the 19th day in the 19-day series where they generally were checked hourly. Offspring and injected mothers were sacrificed by cervical dislocation when the young were 6 months old. The lungs were excised, fixed in Tellyesniczky's fluid, and the tumors in each lobe counted in 70 percent alcohol under a magnification of 12 ×. Surface nodules together with those within the lung parenchyma, which were detected by compressing the tissue between the jaws of a smooth-tipped forceps, were both included in the totals. Questionable masses, together with tumor nodules selected at random, were sectioned, stained with hematoxylin and eosin, and the diagnoses confirmed histologically.

For those mice removed by caesarean, the following procedures were employed. Pregnant females were injected with 0.25 cc. of the urethan solution on the 19th day following the discovery of a plug. The females were sacrificed by cervical dislocation 1 or 5 hours following the injection and the fetuses quickly removed. Each was dried thoroughly, dropped several times from a height of about 8 cm., and placed in a petri dish which was covered with a glass funnel. A constant stream of 95 percent oxygen and 5 percent carbon dioxide was introduced through the neck of the funnel while dish and funnel were both agitated. The area surrounding the petri dish was warmed using a 50-watt incandescent light bulb. After a half-hour exposure, the young were removed and fostered on an untreated nursing mother of the same strain whose own litter had been removed previously. The foster mother employed either had littered on the same day or 1 day earlier.

EXPERIMENTAL PROCEDURES AND RESULTS

A total of 187 normal, noninjected controls, 120 pregnant females injected with urethan, and 440 of their offspring were included in these experiments. Only mothers and young alive 6 months after parturition, however, have been considered in the data in table 1.

TABLE 1.—*Incidence and multiplicity of lung tumors in urethanized mothers and their offspring. Mothers injected 17, 18, and 19 days following discovery of vaginal plug.*

Series	Injection-parturition interval	Total mice	Sex	Mice with tumors	Average tumors per lung, all mice	Average age at death, all mice
	Days	*Number*		*Percent*	*Number*	*Days*
A—offspring	17–19	29	♂	62.1	0.9	180
	17–19	40	♀	52.5	.9	180
A—totals	17–19	69	♂+♀	56.5	.9	180
A—mothers	17–19	19	♀	94.7	9.5	353
B—offspring	18–19	40	♂	77.5	2.9	180
	18–19	43	♀	88.3	2.4	180
B—totals	18–19	83	♂+♀	83.1	2.7	180
B—mothers	18–19	20	♀	90.0	7.0	328
C—offspring	18–20	10	♂	70.0	3.7	180
	18–20	7	♀	85.7	3.7	180
C—totals	18–20	17	♂+♀	76.5	3.7	180
C—mothers	18–20	5	♀	100.0	12.6	323
D—offspring	19–19	44	♂	95.5	9.5	180
	19–19	53	♀	96.2	9.2	180
D—totals	19–19	97	♂+♀	95.9	9.4	180
D—mothers	19–19	30	♀	100.0	9.2	358
D[1]—offspring	19–19 (2–8 hour)	59	♂+♀	94.9	10.5	180
	19–19 (8–20 hour)	38	♂+♀	97.4	7.6	180
D[1]—mothers	19–19 (2–8 hour)	17	♀	100.0	8.6	368
	19–19 (8–20 hour)	13	♀	100.0	9.9	346
E[1]—offspring, caesarean.	19–19 (1 hour)	71	♂+♀	100.0	19.1	180
E[2]—offspring, caesarean.	19–19 (5 hour)	69	♂+♀	97.1	16.4	180
F—controls	No urethan	177	♂+♀	7.9	.1	180

Females close to term were isolated for subsequent injection with urethan as follows:

Series A:—Twenty-two females, injected on the 17th and littered on the 19th day "post-plug." Nineteen mothers, and 15 litters containing 69 young were alive after 6 months.

Series B:—Twenty-four females, injected on the 18th and littered on the 19th day "post-plug." Twenty mothers, and 19 litters containing 83 young were alive after 6 months.

Series C:—Six females, injected on the 18th and littered on the 20th day "post-plug." Five mothers, and 3 litters containing 17 young were alive after 6 months.

Series D:—Thirty-five females, injected on the 19th and littered on the 19th day. Two of the litters in this series (7 mice) were born within the first 5 hours of the 20th day. Thirty mothers, and 25 litters containing 97 young were alive after 6 months.

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Series E:—Thirty-three females, injected on the 19th day, were sacrificed on the same day and the young delivered by caesarean. Series E^1—71 mice, delivered from 18 females 1 hour following injection; Series E^2—70 mice, delivered from 15 females 5 hours following injection. There were 71 survivors in Series E^1 and 69 in Series E^2 after 6 months.

Series F:—One hundred and eighty-seven nontreated control mice; 177 survived and were sacrificed at 6 months of age.

In the 17- to 19-day group, a lung tumor incidence of 62.1 percent was observed for the males and 52.5 percent for the females. An average of 0.9 tumor per lung was obtained for all the mice in either sex. The mothers averaged 9.5 tumors per lung and had a tumor incidence of 94.7 percent.

In the 18- to 19-day group, a lung tumor incidence of 77.5 percent was observed for the males and 88.3 percent for the females. An average of 2.9 tumors per lung was observed in the males and 2.4 per lung in the females. Ninety percent of the mothers were susceptible and bore, on the average, 7 tumors per lung.

In the 18- to 20-day group, the males had a lung tumor incidence of 70 percent and the females, 85.7 percent. An average of 3.7 tumors per lung was obtained for all the mice in either sex. Five of five mothers were susceptible and bore, on the average, 12.6 tumors per lung.

In the 19- to 19-day group, the males had a lung tumor incidence of 95.5 percent and the females 96.2 percent. On the average, 9.5 tumors per lung were observed in the males and 9.2 in the females. All 30 mothers bore tumors, an average of 9.2 per lung.

Since no sex difference either in tumor incidence or in average tumors per lung was evident in any of the series, totals for each were considered valid for purposes of comparison.

Pregnant females in the 19- to 19-day series were injected with urethan and then examined hourly for the first 8 hours, and at 2-hour intervals thereafter, to determine the time between injection and parturition. Although incidence remained about the same, more tumors per lung were obtained when the time between injection and parturition was 2 to 8 hours as compared with 8 to 20 hours. The mothers in the 19- to 19-day series, on the average, bore 8.6 tumors per lung in the 2- to 8-hour group and 9.9 in the 8- to 20-hour group. A lung tumor incidence of 100 percent and an average of 19.1 tumors per lung was observed among the caesarean-delivered young that had been exposed to carcinogen *in utero* for 1 hour (Series E^1). In the group exposed for 5 hours, Series E^2, tumor incidence was 97.1 percent with an average of 16.4 tumors observed per lung.

Among the control mice, Series F, a lung tumor incidence of 7.9 percent and an average of 0.1 tumor per lung was observed.

DISCUSSION

Injection of pregnant mice once with urethan during the later days of gestation produced lung adenomas in the mothers, and in a high percentage

of the offspring (table 1). When pregnant mice were injected on the 19th day and littered on the same day, significantly more of the young bore lung tumors than did the offspring of those injected on the 18th or 17th day. In addition, a higher tumor incidence was observed among the young from the 18-day series as compared with the 17-day series. Not only did the mice in the 19- to 19-day series have a higher tumor incidence, but they also had a greater number of tumors per lung than the mice in the other litter series. Since this could have occurred if more of the carcinogen reached the fetal lungs during the last day of gestation, a possibility previously suggested by Larsen (*11*), it was considered of interest to see whether a variation of a few hours in the injection-parturition interval during the 19th day would affect tumor incidence or multiplicity. It was observed that although incidence remained about the same, more tumors per lung were obtained in the young born within the first 8 hours, as compared with 8 to 20 hours following injection. These results are not conclusive, however, since the significance is borderline ($P<.05$). A greater number of lung tumors also was observed among the young in the 18- to 19-day as compared with the 17- to 19-day series. The present experiments, therefore, confirm those of Larsen (*11*) in that incidence and multiplicity of lung tumors are significantly greater in offspring born of pregnant females injected with urethan when the injection-parturition interval is only 1 day as compared with 2 or more days. This has now been observed not only for pregnant females injected on the 18th day which littered on the 19th day, the one day group of Larsen, but also for pregnant females injected still closer to term, on the 19th day. In similar experiments using pregnant strain C mice, Smith and Rous (*13*) observed no such difference in tumor susceptibility with time of injection when the young were sacrificed 10 days or 60 to 70 days after birth. Although the reasons for this discrepancy are not known, a contributing factor may have been the repeated use of urethan, some of the mice receiving as many as six injections. Smith and Rous (*13*) reported that such mice did not do well and that their offspring were much below weight. Sixteen offspring were included in the 10-day group, some receiving 4 injections while others received 6. In the 60- to 70-day group, 49 young were employed and the number of injections varied from 1 to 6. In view of differences in number of injections of urethan, period of observation, strain, and number of animals employed, a comparison of the results of Smith and Rous (*13*) with those we obtained or with the observations of Larsen (*11*) may not be justified.

Tumor incidence and number of tumor nodules per lung in the 18- to 19-day series did not differ appreciably from that seen in the 18- to 20-day series. This was unexpected, for more tumors were anticipated when the injection-parturition interval was 1 day as compared with 2. Over 90 percent of the pregnant mice littered on the 19th day "post-plug." Thus, the offspring of mice in the 17- to 19-day group could have been exposed *in utero* to urethan and/or its metabolites for about 2 days, and in the 18- to 19-day group for about 1 day. Since the cages containing mice

in the 18- to 20-day group were checked once for litters, on the morning of the 19th day and similarly on the 20th, it is possible some of the young may have been born during the afternoon or early evening of the 19th day. In view of this and because of the small number of observations in the 18- to 20-day series (17 mice from 3 litters), no definite conclusions are warranted.

More tumors per lung were observed in the offspring of Series E, the caesarean series, than in those of the other series (table 1). This was true even for Series D^1 where the pregnant mice also were injected on the 19th day and then littered hours later. Although the reason for this increase in tumor susceptibility in the caesarean series is not apparent, this could have occurred if the fetal lungs were exposed to a greater amount of the carcinogen. A number of operative procedures may have contributed more or less to this condition. Following injection with urethan, pregnant mice in Series E were sacrificed by cervical dislocation. The anoxemia thus produced in each fetus probably helped to stimulate blood flow and to favor the aspiration of amniotic fluid. Both may have produced an increase in the total intake of urethan. A similar influence probably was exerted during the caesarean operations as a result of operative handling, and exposure of uterine horns and fetuses to the air. Following the removal of each fetus, it was quickly placed in 95 percent oxygen: 5 percent carbon dioxide for one-half hour during which time heat was applied and the mice agitated. Although these procedures may have affected fetal lung metabolism as well as the metabolism and elimination of urethan, their precise role in lung tumorigenesis is not known.

Following the injection of pregnant females with urethan, it was expected that more tumors would be induced in them than in their offspring since the adult lungs would have been exposed to a greater quantity of carcinogen. Although this was observed by Larsen (*11*) and in the present experiments (series A, B, C), the reverse occurred in Series E containing the caesarean-delivered mice. In Series D, the offspring bore about as many lung tumors as the mothers. It is possible that other factors are involved besides those concerned with increasing the amount of urethan reaching the fetal lung. Thus, the fetal lung may be more susceptible than the adult lung to urethan. Smith and Rous (*13*) injected pregnant mice with this carcinogen and observed adenomas even among offspring 3 days of age. The possibility exists, too, that the rate of metabolism of urethan is different in the fetus than in the adult. Thus, effective concentration of carcinogen per gram of tissue may be higher in the fetus.

It would appear that fetal lungs were exposed to carcinogen *in utero* longer in the 5-hour than in the 1-hour caesarean series. Although the rate of metabolism of urethan in pregnant mice is not known, it has been reported for intact, nonpregnant mice that approximately 90 to 95 percent of urethan remains after 1 hour and 60 to 73 percent after 5 hours (*15,16*). Despite this, about as many tumors per lung were observed in the 1-hour as in the 5-hour series (table 1, series E^1, E^2). Larsen (*11*) injected pregnant mice once with 25 mg. of urethan 1 day before term and observed

more tumors per lung when injection was intravenous rather than intraperitoneal. He suggested that this may have been due to a relatively higher peak concentration of urethan in the lung in the intravenous group. Induction of approximately the same number of lung tumors in the 1- and 5-hour caesarean groups also may be explainable if the initial, high concentration of urethan which reached the fetal lungs, and which probably was the same in both groups, was the determining factor.

Urethan is a highly diffusible compound. It has been observed within a short time following intraperitoneal injection in mice (*15*, *16*), or rats and rabbits (*17*), that urethan is rapidly distributed throughout the tissues with no appreciable localization. In the present experiments, the pregnant mice used in the caesarean series weighed on the average 37 gm. The fetuses that were delivered averaged 1 gram. Assuming the mouse placenta to be permeable to urethan and that this compound was distributed in a similar pattern throughout both fetus and adult, the amount reaching the fetal lungs would probably be much less than 1 mg. Urethan is metabolized rapidly in the intact mouse. Thus, among mice injected with carbonyl-labeled urethan where the respiratory carbon dioxide was collected continuously for 24 hours, 90 to 99.7 percent of the total C^{14} activity was recovered (*15*). In two different experiments by the same authors, 4.7 and 9.7 percent of the total activity were recovered after 1 hour, and 27 and 40 percent after 5 hours. Following caesarean delivery and the onset of respiration, the small amount of urethan which had crossed the placenta probably was subjected to metabolism, so that the initial concentration would have declined. The fact that an average of 19.1 tumors was observed in fetal lungs exposed for only 1 hour *in utero* suggests urethan is a more potent carcinogen than has heretofore been suspected. Experiments in progress indicate that a much smaller time exposure also is effective. The observation that a carcinogen such as urethan may pass from mother to fetus across the placenta and induce tumors in the young is of prime importance. Thus, if naturally occurring carcinogens were formed, even temporarily, during the later stages of pregnancy and then crossed the placenta, this might account for some of the tumors which subsequently appeared in the offspring.

SUMMARY

Pregnant albino mice related to strain A were injected once with urethan on the 17th, 18th, or 19th day following the discovery of a vaginal plug. Although most of the mice were allowed to litter, some that had been injected on the 19th day were sacrificed, and the young delivered by caesarean 1 hour or 5 hours thereafter. Both offspring and mothers were sacrificed after 6 months when it was observed that the young born following an injection-parturition interval of 1 day had a higher tumor incidence and bore more tumors per lung than those born after a longer interval. Tumor multiplicity in the young was greatest in the 19th-day-caesarean-delivered mice, the 1- and 5-hour groups being about the same.

Urethanized pregnant mice which littered bore more tumors per lung

than their offspring, except when injection was on the 19th day. Here, tumor multiplicity was about the same in both mothers and young. When the young were delivered by caesarean, more lung tumors were observed in them than in any of the mothers.

It would appear from these experiments that urethan is a potent lung carcinogen and that the elaboration of a carcinogen by a pregnant mouse, even for a short time, near term, may induce tumors in the young by penetrating the placental barrier.

A number of conditions are considered that may have contributed to the increased tumor multiplicity observed in mice born after an injection-parturition interval of 1 day or less.

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INDUCED ADAPTATION IN A TUMOR: PERMANENCE OF THE CHANGE [1]

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A previous paper (*1*) reported that when a tumor which originated in an inbred mouse was transplanted into an F_1 hybrid host a change in the transplantability of the tumor occurred. Briefly stated the observation was that when the tumor was transplanted from the strain of origin to resistant backcross hosts it grew progressively in only a few of the latter; however, when the tumor was first grown in an F_1 hybrid and then transferred to resistant backcross hosts a threefold increase in its ability to grow there was observed. The change in transplantability did not appear to be a fortuitous or random mutation such as had been previously reported by others [cited in (*1*)]. On the contrary, the same change could be induced repeatedly (all of more than 20 trials) in transplants from an apparently unchanged stock tumor which was maintained in the strain of origin.

The fundamental basis or nature of the change was not apparent and, as an aid to interpretation, we wished to learn whether the change in the potentialities of the tumor tissue was a permanent or a transient one and, if permanent, whether it became so during growth in hybrids or only after residence in backcross hosts. Experiments designed to throw light on these questions have been completed and are reported herein.

MATERIALS AND METHODS

The technical details in this work are similar to those described in the previous paper (*1*); therefore only the major features are given herein.

The general outline of the experiments was as follows: In the previous work the ability of a certain tumor to grow progressively in resistant backcross hosts was observed under 2 sets of conditions, namely, when it had previously grown only in the strain of origin, and, secondly, when it had previously grown in an F_1 hybrid host. In the first case the tumor grew in 8 percent of backcross animals, whereas in the second case it grew in 30 percent. From one of these latter tumors an "adapted" subline of the tumor was established and maintained by transplanting from backcross to backcross. For simplicity the tumor that had been

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maintained continuously in the strain of origin is called "stock" tumor, the tumors that had been transferred serially in F_1 hybrids during the previous work are called the "1st subline," and those that were maintained by transplanting from backcross to backcross are called the "2d subline." The 2d subline was observed for constancy in its transplantability, and from it a 3d subline was established. The 3d subline comprises tumors that were taken from the 2d, or adapted, subline and returned to the strain of origin for one or more generations to see if that procedure would reverse the change in transplantability. The 4th subline comprises tumors taken from stock, grown in F_1 hybrids for 1 generation, returned to the strain of origin for 3 or more generations, and then tested for their ability to grow in backcross hosts. The last procedure was done for the dual purpose of finding whether a single generation of growth outside the strain of origin was sufficient to produce a relatively permanent change in transplantability of the tumor and whether the interposition of a backcross host was necessary for the demonstration of any of the effects seen. With all tumors the final test was to transplant into resistant backcross hosts and observe the number of animals with progressively growing tumors. These methods are given in detail below.

Three types of hosts were employed, (a) strain C3H, (b) (C3H $\times$ C)F_1 hybrids produced by mating strain C3H females with strain C (B alb C) males, and (c) (C3HC $\times$ C)BC resistant backcross mice derived by mating (C3H $\times$ C)F_1 hybrid females with strain C males. It should be noted that in these animals the maternal line, in the case of hybrids, and the grandmaternal line, in the case of backcross mice, were always strain C3H, and the paternal line was strain C. In the data this distinction is maintained when making comparisons with the previously published results.

The tumor, a mammary adenocarcinoma called C3HBA, has been described previously (*2*). It originated in strain C3H and grows progressively in all strain C3H and F_1 hybrid mice used here. It has never grown in a strain C mouse (398 mice tested). The stock tumor, which is maintained in strain C3H mice, was in the 104th transplant generation at the end of this work and had undergone no apparent change since it was originally described. In addition to earlier tests, it was transplanted into strain C mice at the 70th, 93d, and 104th generation and grew in none (60 mice tested). As another check against the possibility that a spontaneous enhancement in the transplantability of the tumor might have occurred, the stock tumor was transplanted into resistant backcross animals at the 69th and 102d generations and no such enhancement was found (3 tumors in 133 hosts).

Tumor tissue of the 1st subline pertains primarily to the previous experiments but it furnished the source of the 2d subline. At the 7th generation (in F_1 hybrids) of the 1st subline the tumor was, as usual,

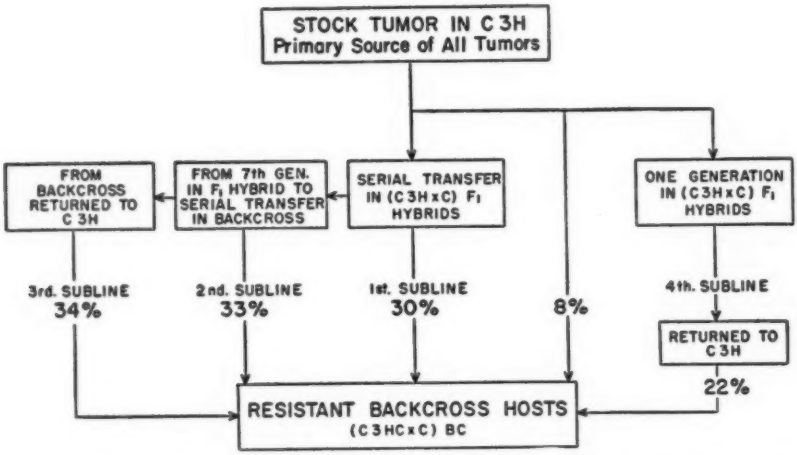
transplanted into backcross animals. It grew in 4 of 10, and 1 of the 4 furnished tissue for the establishment of the 2d subline. This adapted subline was maintained through 30 generations by routinely transplanting it into small groups (usually 10) of backcross animals, and a record was kept of the number of animals in each generation that died with the tumor.

The 3d subline of tumors comprised tumors taken from the 2d subline, transplanted to strain C3H hosts for 1 or more generations, and then tested for transplantability into backcross hosts. Three independent but similar experiments were done in this category; at the 9th, 13th, and 17th generations of the 2d subline a single tumor was selected at random and transplanted into a small group (usually 10) of strain C3H hosts, where it grew in all animals. In each experiment the tumor was grown for 5 successive generations in strain C3H hosts. From each of the 5 generations a tumor was selected at random for transplantation into the next generation in strain C3H, and at the same time it was inoculated into backcross mice to test its ability to grow there.

The 4th subline of tumors was derived by taking stock tumor (*i. e.*, unadapted) from the 90th generation in strain C3H and inoculating it into a small group of F_1 hybrid hosts, where it grew in all. After this single period of growth in F_1 hybrids a tumor was picked at random and returned to strain C3H hosts, where it grew in all and was maintained for 5 successive generations. These tumors were transferred and tested in a manner similar to that described for the 3d subline, except that in this case testing in backcross animals was omitted at the 1st and 2d generations in C3H to conserve the relatively small supply of backcross animals. The experiment was repeated in detail with unadapted tumor taken from the 94th generation of stock tumor.

In the case of each subline of tumors a record was kept of the growth of the tumors in backcross hosts and the data are based upon the number of animals in which the tumor grew progressively and was present at the time of death. In the work with the 2d subline there is a minor exception to the last statement. In each generation of tumor there is one animal, recorded as positive, that was sacrificed to furnish tissue for transplantation. The tumors selected for this purpose were medium sized tumors that were both older and larger than any tumor that had been observed to regress. Thus it seemed quite safe to assume that these animals would have died with tumors; however, if these animals were excluded from the data the conclusions would be unchanged.

Text-figure 1 is furnished as an aid in visualizing the relationships of the sublines of tumors. The genealogic relationships shown are those of the tumors and not those of the host animals. All tumors have a common origin in tumors of the stock line, and all were finally tested for transplantability into a uniform population (uniform as to populations, not as to individuals) of backcross mice.



TEXT-FIGURE 1.—Percentage of tumors that grew progressively in backcross hosts after varied transplantation histories for the tumor. The position of the boxes and the arrows indicate the genealogies of the tumors and not that of the hosts. The tumors grew in all the strain C3H and F_1 hybrid hosts regardless of the source of the inoculum. The four sublines of tumor referred to in the text are shown. Note that for all tumors the primary source and the ultimate host are the same.

RESULTS

As was seen in the previous work, the results in small groups of hosts tend to vary at random about a mean. However, no significant deviations between the groups in a single subline were observed, and for simplicity the groups are aggregated in the data shown in table 1. In this table the first two lines show the data from the previous work for purposes of comparison.

TABLE 1.—*Results of inoculating mammary adenocarcinoma* C3HBA *into resistant backcross mice after exposing the tumor to varied intermediate hosts. The results for each subline are aggregated from several experiments. See text for full description of the sublines*

Transplantation history of the tumor previous to testing in backcross hosts	Results in backcross hosts		
	Number inoculated	Number of tumors	Percent positive
(1) Direct from stock tumor in C3H	182	15	8
(2) From C3H to F_1 hybrid for a varied number of generations (1st subline)	86	26	30
(3) As in (2) then serially in backcross (2d subline)	427	142	33
(4) As in (3) then in C3H (3d subline)	163	56	34
(5) From C3H to F_1 hybrid for 1 generation then returned to C3H (4th subline)	219	48	22

Tumor tissue of the 2d subline (*i. e.*, transferred serially in backcross hosts) did not revert to the lower degree of transplantability at any time during the 3 years represented by the 29 generations that were complete at

the close of these experiments. Aggregated results for the first 10 generations show 40 lethal tumors in 120 backcross hosts, a percentage of 33. Aggregation of the last 10 generations shows 48 tumors in 164 hosts, or 29 percent. The over-all results in 29 generations were 142 tumors in 427 hosts, or 33 percent (table 1—line 3). The 29th generation alone had 4 tumors in 14 hosts, or 29 percent; and finally, the 30th generation, in which some animals are still alive as this is written, indicates a result that will be the same or higher.

Transplanting the tumor tissue of the 3d subline (*i. e.*, adapted tumor returned to C3H hosts) back into the strain of origin for 5 generations (about 17 weeks in all) did not result in a reduction of its ability to grow in backcross hosts. After 1 generation in C3H hosts transplantation to backcross animals resulted in 4 tumors in 17 hosts, or 24 percent. Similarly, after 2 generations of growth in C3H transplantation to backcross resulted in 11 tumors in 22 hosts, or 50 percent. Corresponding figures for 3, 4, and 5 generations in strain C3H hosts are: 8 tumors in 23 hosts, or 35 percent; 16 tumors in 55 hosts, or 29 percent; and 17 tumors in 46 hosts, or 37 percent. The over-all results were 56 tumors in 163 hosts, or 34 percent (table 1—line 4).

Tumor tissue of the 4th subline (*i. e.*, in F_1 hybrid for 1 generation then returned to strain C3H hosts) gave results which were somewhat less numerically but still obviously of the same order as the adapted tumor and not like the stock tumor. After the 3d, 4th, and 5th generations in strain C3H hosts the results of transplantation into backcross hosts were 12 tumors in 60 hosts (20 percent), 15 tumors in 78 hosts (19 percent), and 21 tumors in 81 hosts (26 percent), respectively. The over-all results for this subline were 48 tumors in 219 hosts, or 22 percent (table 1—line 5). The statistical analysis of these results and that of the other results are mentioned in the discussion.

DISCUSSION AND CONCLUSIONS

It is a matter of common knowledge that the biologic system represented by a tumor growing in a mouse is a very complex one involving many variables and not apt to yield results that are ideally uniform. The results presented here exhibit such irregularities but they should not be allowed to confuse the issue. The point to be decided was not whether each of the sublines was exactly like the others in all respects but rather whether a major difference existed between the stock tumor and all the sublines, whether the differences were of the same order, and whether the differences could be abolished by returning the adapted tumor to the strain of origin. In other words, one wished to discover whether a given subline was the sort that would grow in approximately 8 percent of backcross animals or of the sort that would grow in three times that many. From that viewpoint the results are quire clear. Tumors of each of the four sublines are obviously different from the stock tumor, and the differences are highly significant statistically ($P<0.001$ in each case). Furthermore, in neither the 2d, 3d, or 4th subline is the result significantly

different from that previously obtained for subline 1. (P is between 0.9 and 0.8 for the 2d and 3d sublines and between 0.2 and 0.1 for the 4th subline).

When the adapted tumor was maintained in backcross hosts for a long period of time it did not revert to its original state and it was not caused to do so by five generations of "reconditioning" in the strain of origin. Even after a single generation (3 weeks) in F_1 hybrids followed by five generations (15 weeks) in the strain of origin the tumor was found to be in the adapted state. Thus the "adaptation," whatever its true nature may eventually prove to be, represents a change in the properties of the tumor that is relatively permanent and that becomes so during a single generation of growth outside the strain of origin. The main effect of this, with regard to final interpretation of the phenomenon, is that these results do not rule out the possibility that the change is a mutation, as they might have done if the change had proved to be transient. However, neither do they prove that the change is a mutation, and we shall continue to refer to it as an "adaptation."

Something more should be said regarding the statistical aspects of the results. It was noted before that each of the sublines differs significantly from the stock tumor and that neither of the three sublines involved in the recent work differs significantly from the 1st subline previously used. However, the result obtained with the 4th subline (22 percent) is statistically different from the results obtained with the 2d and 3d sublines (33 and 34 percent); in both cases $P<0.01$. This does not destroy the validity of the conclusions relating to the primary purposes of the work but it does raise other questions. There are undoubtedly many influences at work in this material, and it is unlikely that any of the percentages obtained are more than close approximations. So-called biologic variation may be the complete explanation of the variations between sublines but it is possible that, in addition to the major change under investigation, lesser changes take place during long periods of growth outside the strain of origin. Incomplete data collected in this laboratory suggest that such might be the case, and for the time being it seems best to defer judgment as to whether the tumors in the 4th subline are exactly like those in the other sublines with regard to transplantability.

It is interesting to note that there is fairly good agreement between genetic theory (*3*) and the results obtained with tumors of the 4th subline. From a statistical standpoint, 48 positive cases in 219 animals are compatible only with a 2-"factor" tumor (25 percent expected) and do not differ significantly therefrom. This can be compared to the results obtained with stock tumors (15 positive cases in 182 animals) where the data are compatible with either a 3- or a 4-"factor" tumor but not compatible with 2 "factors." When it is remembered that of the sublines this 4th subline has the least complicated history of transplantation the agreement takes on added weight.

These results confirm the previous observations and show that the change in the tumor tissue is of a relatively permanent nature, which

does not depend upon "fixation" by more than one generation of growth outside the strain of origin of the tumor. The data are not sufficient for forming conclusions as to the underlying mechanism of the change, and the possibilities for interpretation discussed in the previous paper remain open.

SUMMARY

It has been demonstrated previously that an enhancement of the transplantability of a tumor could be induced by growing it in F_1 hybrid hosts prior to transplantation into resistant backcross hosts. The experiments reported herein were done to determine whether the change in the potentialities of the tumor tissue was relatively permanent or transient and whether it occurred during the first generation of growth outside the strain of origin of the tumor or required something more. It was observed that the adapted subline of tumor maintained its high degree of transplantability during 30 generations of growth in backcross hosts and did not revert to the low transplantability of the stock tumor from which it was originally derived. Concurrently, the stock tumor did not alter in its low transplantability. An attempt to "recondition" the adapted tumor by growing it in the strain of origin for 5 generations resulted in no lowering of the high transplantability. Finally, a tumor subline which was taken from stock tumor, grown for a single generation in an F_1 hybrid host, and then returned to the strain of origin for 5 generations was found to have the higher degree of transplantability that characterizes the adapted tumor sublines. Thus, within the limits of these experiments, the change in transplantability is induced by a single exposure to the foreign environment and it is a permanent change in the character of the tumor tissue. The fundamental mechanism of the change was not determined.

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TUMORS OF THE GLANDULAR STOMACH INDUCED IN RATS BY INTRAMURAL INJECTION OF 20-METHYLCHOLANTHRENE[1]

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The rat, in common with other laboratory, domestic, and wild animals rarely develops a malignant gastric neoplasm and, moreover, if this lesion does occur it is likely to be carcinoma of the forestomach or of the rumen or sarcoma rather than adenocarcinoma. Thus, spontaneous adenocarcinoma of the glandular stomach is even rarer than a cursory check of the titles in the literature appears to indicate. Bullock and Curtis (*1*) examined a series of 33,000 rats of various ages *post mortem*, and found that only 1 had gastric adenocarcinoma, but with no metastases; 9 animals had gastric sarcoma. McCoy (*2*) examined nearly 100,000 wild rats finding 103 tumors, none of which appeared to be primary in the stomach. Wooley and Wherry (*3*) found that in a series of 23,000 wild rats with 22 tumors, none was a gastric carcinoma. Ratcliffe (*4*) found no gastric tumor among 273 spontaneous tumors of the rat. Saxton *et al.* (*5*) reported a study of 234 neoplasms occurring in 184 of 498 albino rats of the Osborne-Mendel strain. Of these there were 1 squamous carcinoma and 1 polypoid adenoma of the stomach, but no example of gastric adenocarcinoma was found. Of the many rats coming to autopsy at the National Cancer Institute during the past several years, none has shown spontaneous gastric adenocarcinoma.

While the rarity of adenocarcinoma of the glandular stomach of the rat precludes a study of the spontaneous lesion, it lends significance to any such lesion induced by experimental procedures. Attempts to induce gastric carcinoma experimentally in various animal species has been reviewed elsewhere [Klein and Palmer (*6*), Barrett (*7*), and Stewart (*8*)]. Therefore, only brief reference is made to those reports dealing with the rat.

Voronoff and Alexandrescu (*9*) reported the production of a metastasizing adenocarcinoma of the stomach of the rat by oral administration of coal tar, wool fat, aniline oil, and toluenediamine. We agree with the opinion expressed by Klein and Palmer (*6*) that the evidence that Voronoff

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and Alexandrescu induced carcinoma is not convincing. Roffo (*10*) reported the induction of adenocarcinoma of the stomach of the rat following the oral administration of heated fats, oxidation products of cholesterol, and a distillate of fuel oil. Although it appears to us after an examination of his profusely illustrated publications that his diagnosis of gastric adenocarcinoma was not warranted, many of his animals exhibited severe ulcerative gastritis and a few, genuine sarcoma of the stomach. Bullock and Rohdenbrug (*11*) introduced spine balls into the lumen of the glandular stomach of rats and produced a lesion that they described as cystadenoma. Their interpretation of the pathogenesis of this process was the imperfect healing of ulcers, due to chemical and mechanical irritation produced by the spine balls. Rusch *et al.* (*12*) opened a new line of attack upon the problem by the intramural injection of 3,4-benzpyrene in corn oil. A lesion interpreted as adenocarcinoma occurred in 1 of 5 rats so treated. The specimen was not illustrated in their report. Klein and Palmer (*6*) rejected this report because of inadequate pathologic data upon which to base a diagnosis of carcinoma. Barrett (*7*), on the other hand, accepted it as the first *bona fide* example of experimental induction of carcinoma of the glandular stomach. In view of the findings of the present experiment, we are inclined to regard the experimental gastric lesion induced by Rusch and his associates as an example of the adenomatous diverticulum described below. Howes and de Oliveira (*13*) introduced threads impregnated with 20-methylcholanthrene into the wall of the glandular stomach of rats. Approximately 150 animals were killed at different intervals thereafter over a period of 590 days. These experiments were designed primarily to study the evolution of neoplastic changes in the tissues in the region of the thread. It is difficult to be certain how many of the resulting gastric lesions were diagnosed as carcinoma by Howes and de Oliveira and what criteria were used by them in arriving at this diagnosis. The authors state that in 2 animals large tumors grew outside the stomach and there were peritoneal implantations locally, but no metastases in liver or lung. Presumably, these 2 lessions were considered to be carcinoma. There were also 7 sacomas, 2 of which were combined with epithelial growths.

MATERIALS AND METHODS

In the present experiment, 300 rats, 2 to 10 months of age and of 3 strains, were used: the Osborne-Mendel; the Marshall 520; and the AxC 9,935, sometimes referred to as the "Irish" strain or simply the AxC strain. They were fed Purina dog chow and given water *ad libitum*. Under intraperitoneal nembutal anesthesia, the stomach was delivered through a midline incision. Using a sterile tuberculin syringe, a dose of 0.02 cc. of an aqueous suspension containing 0.6 mg. of 20-methylcholanthrene (table 1) was injected into the submucosa at each of 2 sites in the anterior wall of the glandular stomach. The first injection-site was the wall of the antrum near the pylorus. The second injection-site was the wall of the acid-secreting or fundic portion of the glandular stomach at

a point not less than 5 mm. distal to the limiting ridge. The technical success of the injection was evidenced by the presence of a small yellow bleb at the site of injection (fig. 1). Following operation, the animals were examined from time to time for the presence of palpable gastric tumors. Some of the rats were treated with penicillin as soon as they developed a palpable gastric tumor with the hope of controlling gastric infection and thereby prolonging life so as to afford an opportunity for local spread and metastasis of the neoplasm.

TABLE 1.—*Composition of aqueous 20-methylcholanthrene suspension*

	Grams percent
20-methylcholanthrene	3. 0
Aerosol O T	. 01
Methocel (4,000)	. 2
Ethyl alcohol	1. 0
Distilled water: *q. s.*100.	
pH adjusted to 7.4 with 0. 1 N NaOH prior to making up to volume with water.	

A total of 35 animals was discarded from the experiment because they died during or immediately after operation or, dying later, they showed post-mortem changes that interfered with the histologic interpretation of the lesions. The remainder of the animals, 265 in number, were allowed to live as long as possible unless they showed signs of impending death; these were killed and autopsied. In the final analysis, 103 male and 10 female strain OM rats, 49 male and 37 female strain AxC rats, and 2 male and 64 female strain M–520 rats were examined *post mortem*. At autopsy in some animals small portions of any gastric tumor present were removed, and bits of this tissue were immediately inoculated subcutaneously, intramuscularly, or intraperitoneally into young rats of the same strain. The esophagus and duodenum were ligated, and the stomach was distended with fixative for 4 to 8 hours after which it was opened and examined for mucosal lesions. Pieces of tissue were obtained for subsequent histologic study. Occasionally, the stomach was opened at the time of autopsy and fixed after pinning flat on a cork board. The origin of the tumors, whether from the antrum, the acid secreting area, or from both sites, was determined as precisely as possible at autopsy. The tissue for histologic study was preserved in Zenker-acetic acid solution, embedded in paraffin, and sections were stained with hematoxylin and eosin. In a number of cases, the periodic-acid Schiff reaction, van Gieson's stain, phosphotungstic acid-hematoxylin stain, and Wilder's silver impregnation method for reticulin were employed.

RESULTS

The most frequent lesion in the present experiment was an adenomatous diverticulum (table 2), which occurred 273 times in 220 rats at one or the other or at both sites of injection. There were 28 malignant tumors in 26 animals as follows: 4 adenocarcinoma, 4 adenoacanthoma, 3 mixed

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carcinoma and sarcoma, 16 sarcoma and 1 unclassified. Of the 16 unmixed sarcomas, 5 occurred at the antral site, 9 at the acid-secreting site, and 2 embraced both sites (table 2).[5]

TABLE 2.—*Lesions induced in glandular stomach of rats by 20-methylcholanthrene injected intramurally into each of 2 sites: the first at the antrum, the second at the acid-secreting area*

Lesion	First site	Second site	Both sites	Total lesions
Adenomatous diverticulum	206	63	4	273
Adenocarcinoma	4	0	0	4
Adenoacanthoma	3	0	1	4
Carcinosarcoma	1	2	0	3
Sarcoma	5	9	2	16
Unclassified malignant tumor	1	0	0	1
No neoplastic response	38	184		
Total	258	258	7	

The longest period of time that any animal lived following injection of the methylcholanthrene was 35 months. Clinically, tumors were first palpated approximately 9 months postinjection. However, in another experiment, animals receiving a similar injection of methylcholanthrene, and killed at weekly intervals thereafter, exhibited grossly visible tumors at autopsy at 12 weeks.

ADENOMATOUS DIVERTICULUM

The adenomatous diverticulum, which is considered to be precancerous, occurred much more frequently at the first or antral injection site (table 2) than at the second injection site. This lesion consists of an adenomatous diverticulum in continuity with the lumen of the stomach, which it often equals or exceeds in size (figs. 2–9, 13–23, and 30). Externally, there is chronic peritonitis with fibrous adhesions to surrounding structures, which grossly may be mistaken for infiltrating carcinoma. Although the development of the lesion is accompanied by thinning of the wall of the stomach, the integrity of the serosa and part of the muscularis propria are maintained. Thus, the most deeply placed glands of the adenomatous diverticulum are separated from the peritoneal cavity and surrounding structures and viscera by a barrier consisting of peritoneum and fibrous inflammatory tissue, in which there are remnants of the muscularis. The fibrous tissue of involved areas and the walls of many of the neighboring arteries show fibrinoid degeneration. The arteries are often partially or completely occluded as a result of chronic arteritis. The adenomatous diverticulum is honeycombed with microcysts and macrocysts, the largest sometimes equaling the lumen of the stomach in volume. These cysts are lined with altered glandular epithelium, and

A number of lesions involving tissues and organs other than the stomach were found at autopsy in animals of this experiment. Since many of the rats had reached an advanced age, it is probable that many of these lesions were spontaneous. These lesions will be contrasted with those occurring in untreated animals of similar age, sex, and strain in a forthcoming article.

many of them contain pultaceous material consisting of mucus and inflammatory exudate and necrotic debris, which are also present within the glands and throughout the stroma. Polypoid adenomatous nodules extend into the cysts and into the lumen of the stomach, and are composed of cells that are chiefly of the mucous variety. Although hyperchromatic and hyperplastic in appearance, these cells do not, in most instances, show the convincing cytologic variations so characteristic of a genuinely malignant neoplastic process. Specimens showing suggestive transitions between the adenomatous diverticulum and carcinoma are shown in figures 15, 20, 21, 22, and 23. The adenomatous lesion first appears as a small submucosal herniation of glandular mucosa. The epithelial cells in this early lesion are, as far as we have been able to determine, exclusively mucous cells. As the lesion enlarges, the features of cyst formation, glandular proliferation, polypoid formation, and inflammation become prominent.

Squamous-cell metaplasia was present in 18 of the lesions of adenomatous diverticulum, despite the absence of direct continuity with the forestomach. It occurred in 16 lesions at the first injection site and in 2 lesions at the second injection site. Hence, while squamous-cell metaplasia occurred 8 times as frequently in the antral site as in the fundic site, this difference may be attributed in part to the fact that a greater number of tumors, as well as much larger ones, occurred at the antrum. The same holds true in regard to metaplastic bone formation in the adenomatous diverticulum, which occurred 12 times in antral lesions, 4 times in fundic lesions, and once in a massive lesion involving both sites. The bony spicules that developed within the stroma were irregular in shape and without marrow, and appeared to be completely benign.

ADENOCARCINOMA OF GLANDULAR STOMACH

There were four cases of adenocarcinoma of the glandular stomach (figs. 10, 11, and 24–29). The most exacting criterion for the diagnosis of these experimental gastric carcinomas is the presence of a neoplastic lesion that infiltrates all coats of the wall of the viscus and forms a tumor mass on the serosa. This was the extent of the lesion in one case (F5615). In another case (G18553), the carcinoma also invaded the liver. In the other two cases (F7311 and G7877), there were secondary neoplastic deposits in liver, pancreas, and regional lymph nodes, and the latter of these cases exhibited in addition intravascular pulmonary deposits. In all four of these cases, the carcinoma was associated with a lesion of adenomatous diverticulum. In the literature, the description of induced gastric adenocarcinoma of the rat is scanty. Therefore, we have given case reports of each of the four rats.

CASE #F5615

A female strain AxC rat, subjected to operation at the age of 4 months, showed emaciation, dyspnea and a palpable mass in the upper abdomen 13 months later. The tumor at autopsy measured 18 by 18 by 4 mm., and involved the glandular stomach from a point beginning 9 mm. distal to the limiting ridge and extending to

the pylorus. It was firm, gray, and nodular on the serosal surface, and ulcerated. Microscopically, the tumor was composed of acini, irregular in size and shape, and lined with cells having relatively large hyperchromatic nuclei. There were widespread invasion of the muscularis propria and extension onto the serosa over a broad area. A part of this serosal nodule was excised at autopsy for transplantation. The excised piece removed from the histologic specimen an area of tissue that was in continuity with the infiltrating carcinoma, and presumably contained neoplastic tissue.

CASE #F7311

A strain Marshall 520 female rat subjected to operation at the age of 2½ months exhibited a palpable epigastric mass 11 months later. At autopsy, a large tumor of the glandular stomach was found arising at the first site of injection (figs. 11, 24, 25). The tumor was nodular, firm and glistening, except in areas where it was covered by adhesions that bound it to liver and pancreas. One of the serosal nodules was excised for transplantation. Adjacent to the posterior surface of the stomach was an enlarged lymph node, and extending from it to the liver were dilated lymphatic channels. At the second site of injection, there was a small cystic lesion not in continuity with the antral tumor. Microscopically, the antral tumor was composed of acini, cords, and nests of neoplastic glandular cells. These cells contained mucus, while cells in other areas possessed scanty amounts of granular cytoplasm free of mucus, and the nuclei were hyperchromatic. The neoplasm infiltrated the muscularis propria extensively, invaded the wall of the duodenum, and had broken through the wall of the stomach to infiltrate the liver and pancreas. Extensive areas of these organs were replaced by tumor. The advancing border of the infiltrating neoplasm was irregular; a few detached tumor deposits occupied spaces resembling dilated perivascular lymphatic channels. The enlarged postgastric lymph node observed at autopsy was almost completely replaced by tumor tissue, smaller deposits of which were present in three other lymph nodes within the pancreas. In the surrounding pancreatic tissue there were several dilated lymphatic vessels containing deposits of tumor tissue.

CASE #G7877

A strain AxC male rat was subjected to operation at 2 months of age and found dead 23 months later. At autopsy, the stomach and liver were firmly adherent to the anterior body wall by dense adhesions which concealed an abscess 4 cm. in diameter (figs. 10, 26, 27, 28, and 29). There was a fistula between the lumen of the stomach and the cavity of the abscess. The wall of the stomach adjacent to the abscess was thick and rigid. Approximately 85 percent of the liver was destroyed by the abscess, the wall of which was composed chiefly of hepatic tissue. A lymph node 1 cm. in greatest diameter was located between the stomach and spleen. Microscopically, the thickened wall of the glandular stomach was covered on its luminal surface by fairly well differentiated glandular structures lined with cells having enlarged hyperchromatic nuclei. Deeper in the wall, the epithelial cells were more irregular, acinous structures were fewer, and the cells grew in sheets, cords, and nests. Mitotic figures were numerous, and many of them large. There was pronounced variation in the appearance of the nuclei, some measuring as much as 30 microns in diameter. Extensive areas of the muscularis propria were permeated by tumor tissue that formed large nodules on the serosal surface, invading the liver. One neoplastic nodule on the gastric serosa was separated from the bulk of the tumor and may have represented a lymph node replaced by tumor tissue, although no lymphoid tissue could be identified in the section. The lymph node lying between the stomach and the spleen was largely replaced by tumor tissue. Several deposits of tumor tissue were found in the pancreas and in the vessels of the lung. An incidental finding in this animal was a focus about 8 by 4 by 4 mm. in size, of interstitial-cell hyperplasia or tumor tissue in the right testicle. Similar lesions, some small and others large, have been observed in a number of animals of this strain. The right adrenal gland was absent.

CASE #G18553

A strain Osborne-Mendel male rat, 9½ months old when subjected to operation, was killed 14 months later, at which time it displayed marked weakness and a palpable abdominal tumor. At autopsy, the glandular stomach was found to be the site of a massive tumor 45 by 27 by 18 mm. that was adherent to the liver. A piece of solitary nodule 10 by 10 by 8 mm. that protruded above the serosal surface of the gastric tumor was transplanted. The vena cava near the liver was thrombosed. One retrogastric lymph node measured 10 mm. in greatest diameter. Microscopically, the gastric tumor was composed of two rather distinctive histologic types of tumor tissue. Most of the tumor tissue consisted of hyperplastic mucous glands (like those of the adenomatous diverticulum) lined with tall columnar cells with basally located nuclei and a large amount of pale cytoplasm that contained mucus. The abundant stroma between these glands was edematous, and showed evidence of inflammatory reaction. The stroma also contained a focus of metaplastic bone. On the side of the lumen, the hyperplastic glands formed polypoid growths. The deeply situated glands where they apposed the muscularis propria formed a relatively smooth line, lacking any tendency to invade discretely. Within some of the deeply placed acini, the cells nearest the muscle were degenerated. The second histologic type of tumor was composed of low cuboidal cells with relatively uniform small nuclei and scanty, deeply stained cytoplasm. The stroma was dense and collagenous, relatively free of inflammatory cells, and contained no deposits of metaplastic bone. Extensive areas of the liver were invaded by the tumor tissue, the advancing margin of which consisted of neoplastic glands embedded in an abundant stroma that was everywhere interposed between the liver tissue and the infiltrating neoplastic epithelium. There were no detached extensions of tumor tissue into the liver, but instead the advancing margin of the growth presented a continuous wavy border. The deepest extension of tumor tissue into the liver was composed of neoplastic cells that contained large amounts of mucus. The second site of injection in the glandular stomach showed two small adenomatous diverticula not noted grossly.

ADENOACANTHOMA

Three tumors diagnosed as adenoacanthoma developed at the first site of injection, and a fourth massive tumor involved both injection sites (table 2). Grossly, these tumors presented the characteristics of the adenomatous diverticulum, forming large palpable masses. They were diagnosed as carcinoma on the basis of neoplastic infiltration of all the coats of the wall of the stomach and of the formation of a neoplastic lesion on the serosal surface of the viscus. The massive tumor involving both injection-sites metastasized to the lung. Histologically, the neoplastic tissue had two components, a glandular element and a squamous element, in some cases proliferating as separate and distinct foci, in other cases intermingled. These tumors were separated from the stratified squamous mucosa of the forestomach by uninvolved glandular mucosa. It is believed that the squamous-cell carcinoma component of these growths developed from the glandular mucosa through the intermediation of squamous metaplasia (fig. 19), which occurred so frequently in the adenomatous diverticula. Indeed, in some of these latter lesions, the cellular atypicality and the degree of proliferation of the metaplastic squamous elements were so pronounced that the lesion appeared to be neoplastic (fig. 30) although it had not spread beyond the confines of the viscus.

SARCOMA

There were 19 lesions diagnosed as sarcoma either alone or mixed with carcinoma; 1 additional malignant tumor was so undifferentiated that it could not be classified with certainty as either sarcoma or carcinoma (table 2). Three sarcomas were growing in close association with proliferating glands, and were considered to be mixed carcinoma and sarcoma; in some areas of these growths, obviously neoplastic glands were supported by sarcomatous stroma. Many of these sarcomas were so undifferentiated that their classification awaits further study. A few of the tumors were osteogenic sarcoma. The sarcomas are illustrated in figures 12, 31, and 32. With sarcoma present the wall of the stomach was thickened by firm gray tissue and the mucosa was intact, except when secondarily infiltrated. The neoplastic tissue extended onto the serosal surface of the stomach forming discrete and confluent nodules, and in some instances infiltrated the pancreas, spleen, mesentery, and chest wall. The sarcomas developed both independently of, and in association with, the adenomatous diverticulum. The sarcomas were firm and solid rather than soft, cystic, and mucoid, as were the lesions of adenomatous diverticulum. The osteogenic sarcomas were composed of areas that presented different histologic patterns. Large parts of the tumor were composed of interlacing bundles of spindle-shaped cells. Other areas were cartilaginous, and the cells here exhibited the variations in size, shape, arrangement, and staining that are characteristic of neoplastic cartilage. Still other areas showed osteoid tissue and spicules of bone, interspersed with neoplastic cells that varied so considerably in appearance that they were indistinguishable from the histologic pattern characteristic of osteogenic sarcoma, primary in the bones of man and other animals.

The observers were on the alert for areas of the early stages of developing sarcoma. A few of the adenomatous diverticula showed areas of atypical spindle cells that were interpreted as probable incipient sarcoma.

TRANSPLANTATION [6]

The data dealing with transplantation are shown in table 3. The only malignant tumor that grew following transplantation was a sarcoma. In view of the failure of the other malignant tumors to grow upon transplantation, this criterion of malignancy so valuable in mice may not be valid in rats of the strains employed. Attempts to transplant adenomatous diverticula failed in all but 1 of 25 cases. Since we are not able to evaluate the significance of this single positive result following transplantation of a fragment of tissue from an adenomatous diverticulum, the details of the circumstances are given in a case report.

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[6] According to Dr. Walter E. Heston of this Institute the rats of strain Marshall 520 and of AxC 9935 have been inbred for more than 20 generations. Primary tumors arising in these rats can be readily transplanted to other animals of the same strain. The Osborne-Mendel strain of rats is not so highly inbred. In the present experiment 26 of the transplants were made in rats on the Marshall 520 or the AxC and 8 in the less highly inbred Osborne-Mendel strain.

TABLE 3.—*Transplantation data of all tumors*

Lesion	Number of donor animals	Recipient animals		
		Number inoculated	Number with tumor	Number without tumor
Adenomatous diverticulum	25	78	[1] 1	77
Adenocarcinoma	3	9	0	9
Adenoacanthoma	3	9	0	9
Sarcoma	2	6	[2] 3	3
Carcinosarcoma	1	3	0	3
Total	34	105	4	101

[1] A squamous-cell carcinoma developed at inoculation site; discussed in text of paper.
[2] All from same donor.

The donor rat in this case had received intramural injections of methylcholanthrene into the two sites in the glanular stomach 21 months before it was killed. At autopsy, an adenomatous diverticulum was present, and pieces of tissue excised from it were transplanted subcutaneously into two rats. One host rat failed to develop a tumor. The other host rat was killed 7.5 months later and had a tumor 1.5 cm. in diameter at the site of transplantation, which proved to be a squamous-cell carcinoma. At this writing this tumor is being propagated by subcutaneous transplantation every 2 months, which is the time required for it to achieve a diameter of 2 or 3 cm. Since this is the first experience we have had with transplanting induced neoplastic lesions of the stomach of the rat, we are in no position to attribute the result herein reported either to a spontaneous tumor, to the growth of the transplanted tissue, or to the induction of a new neoplasm in the host animal by methylcholanthrene, possibly contained within the transplant. It is conceivable that the transplantation of a small focus of tissue like that illustrated in figure 30 could develop into a squamous-cell carcinoma in the recipient animal. However, the long time interval of 7.5 months before it developed into a small carcinoma argues somewhat against this. On the other hand, the rat is notoriously resistant to the induction of cutaneous carcinoma by methylcholanthrene. It would be unusual for a spontaneous squamous-cell carcinoma to be subcutaneous. No final conclusion has been reached regarding the significance of this observation.

DISCUSSION

One of the purposes of this experiment was to determine whether carcinoma of the gastric glandular mucosa, unmixed with other malignant neoplastic elements, could be induced in high incidence by the technique employed. The answer to that question is, "No." Only 4 unmixed adenocarcinomas occurred in 265 animals, an incidence of 1.5 percent. There was a total of only 11 tumors composed of adenocarcinoma in whole or in part, an incidence of 4.2 percent. Hence, the technique described herein cannot be recommended for a study of experimental adenocarcinoma of the stomach in which a large yield of this type of neoplasm is required.

The rat may prove not to be a satisfactory test animal for the study of experimental adenocarcinoma of the stomach.

The lesion diagnosed as adenomatous diverticulum, occurring so frequently in animals in this experiment, could readily be mistaken for carcinoma by the uncritical investigator, because of its size, its adhesion to adjacent structures, and the hyperplastic appearance of its epithelial cells. There is little doubt that the adenomatous diverticulum is precancerous in the sense that it is a lesion which may be occasionally followed by carcinoma. The failure of more of the adenomatous diverticula to give rise to overt carcinoma may be attributed in part perhaps to loss of the injected methylcholanthrene by evacuation into the lumen of the stomach (*vide infra*) following the initial developmental downgrowth of the gastric mucosa into the injection-site. As a consequence of this loss of carcinogen the epithelium of the adenomatous diverticulum is not under the influence of methylcholanthrene continuously after the first stage of its development has been completed.

The adenomatous diverticulum is without exact parallel in human or animal pathology. It has not only no exact counterpart in man, but also has not occurred, according to our own knowledge, in other species of animal such as the mouse, rabbit, guinea pig, or monkey receiving the carcinogenic hydrocarbons orally or by intramural injection into the stomach. Upon encountering this lesion in the gross and microscopically for the first time the tendency is to consider it carcinoma. There are several points, however, that distinguish it from carcinoma. Although the cellular elements of this lesion are hyperchromatic and appear to be hyperplastic, they do not exhibit the wide range of variation characteristic of so many gastric carcinomas. These epithelial elements are confined within the attenuated gastric wall, which is composed of fibrous inflammatory tissue and remnants of muscularis propria. This encapsulating barrier in most instances effectively prevents the escape of the atypical glandular elements, and consequently invasion of adjacent organs and tissues does not occur. There is invariably a pronounced inflammatory reaction of stroma, glands, and cystic structures. None of the 273 lesions diagnosed as adenomatous diverticulum yielded metastases, despite their frequent presence clinically for several months and their large size at autopsy. Specimens in which a distinction between the adenomatous diverticulum and carcinoma was made with difficulty are shown in the illustrations. The failure (with one possible exception) of transplants of 25 of the lesions to grow in recipient rats loses some of its significance in view of the low incidence of positive results following transplantation of the carcinomas and sarcomas (only 1 malignant tumor grew of 9 transplanted).

Squamous metaplasia of glandular mucosa and metaplastic bone formation in the stroma of the adenomatous diverticulum are of interest, particularly in relation to two histologic types of malignant neoplasm occurring in this experiment; namely, adenoacanthoma and osteogenic sarcoma. The possibility that the squamous elements in the adenoacan-

thomas were derived from the mucosa of the forestomach was eliminated by the failure to find any direct continuity between this chamber and the adenomatous diverticula. Indeed, squamous metaplasia was relatively more frequent in the adenomatous diverticula primary at the antrum, than in those in the acid-secreting portion of the glandular stomach; thus this is inversely proportional to the proximity of the respective injection sites to the squamous-lined forestomach. The relationship between the areas of squamous metaplasia and the malignant squamous-cell elements of the adenoacanthomas should prove to be a problem worthy of further study.

Metaplastic bone in the adenomatous diverticula was relatively about as frequent in these lesions situated at either of the two injection sites in the glandular stomach. Bullock and Rohdenburg (*11*) described similar bony spicules in their "cystadenomas." What relationship exists between these deposits of metaplastic bone and osteogenic sarcoma in the present experiment is open to future investigation. The present technique can be added to other techniques that elicit osteogenic sarcoma following injection of carcinogenic hydrocarbons into soft tissues of animals. Shimkin and Mider (*14*) reported five bone-forming tumors in the subcutaneous tissue of guinea pigs at the site of injection of methylcholanthrene. Dunning, Curtis, and Bullock (*15*) obtained two subcutaneous "osteochondrosarcomas" similarly induced in the rat by benzpyrene. Dunning and Curtis (*16*) described the development of bone-forming tumors in the rat in cysticercus-induced neoplasms as well as in those neoplasms arising in the subcutaneous tissues at the site of benzpyrene injection. In the mouse, Stewart, Hare, Lorenz, and Bennett (*17*) obtained osteogenic sarcoma in the glandular stomach following intramural injection of methylcholanthrene. Examples of bone-forming tumors in soft tissue of man are not rare. They have been reported in the skin, breast, muscle, connective tissue, bladder, and kidney. In the gastrointestinal tract an ossifying myoma of the stomach (*18*) and several cases of ossifying carcinoma of the colon (*18, 19, 20, 21, 22*) have been described. For discussions of possible mechanisms of pathogenesis of these lesions the reader is referred to the papers by Mallory (*23*), Binkley and Stewart (*24*), and Allen (*25*).

About 70 percent of gastric carcinomas in man involve the antrum. It is therefore especially significant that in the present experiment the majority of the experimental carcinomas and adenomatous diverticula occurred at the antral injection site rather than at the second or acid-secreting site; this despite the fact that both areas were exposed to the same dose of carcinogen simultaneously in the same animal. The epithelial tumors of the antrum as compared with those of the acid-secreting portion of the glandular stomach not only occurred more frequently, but appeared earlier and enlarged more rapidly. The question arose whether these differences were due to resistance of the acid glandular mucosa to neoplastic transformation, to disappearance or inactivation of the methylcholanthrene at the second injection site, or to other factors.

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Autopsy studies disclosed, surprisingly enough, that the injected methylcholanthrene disappeared relatively early from the antral site (or at least it was not identified in many specimens), whereas it frequently remained visible for many months at the second site. In order to confirm this observation methylcholanthrene was injected into the wall of the glandular stomach of another group of rats at the same two sites. The rats were killed at intervals, thereafter, and the respective injection site was excised and transplanted into the subcutaneous tissue of a C3H mouse as a biologic test for the presence of methylcholanthrene (*26*). With methylcholanthrene present in the transplant and not altered chemically, it should produce subcutaneous sarcoma in the highly susceptible recipient C3H mice. This study revealed that the second or acid glandular injection site from the rat produced sarcoma in 24 of 28 recipient mice as compared with only 5 sarcomas in 17 recipient mice inoculated with the first or antral injection site from the rat. Moreover, sarcoma developed in mice inoculated with the acid glandular site (second injection site) from rats injected with methylcholanthrene 20 weeks previously. By contrast, no transplants of antral injection site (first injection site) induced sarcoma in mice if the methylcholanthrene had been injected into the gastric tissue of the rat 12 or more weeks previously. In still another experiment (unreported) a mixture of 20-methylcholanthrene and ground glandular mucosa from a rat stomach was injected subcutaneously into mice. The admixed mucosa did not inhibit the induction of sarcoma in the subcutaneous tissue of mice by methylcholanthrene. These results indicate that the methylcholanthrene with its carcinogenic potency unimpaired, persists in the wall of the acid-secreting portion of the stomach longer than at the antral site. There is therefore greater resistance to the development of carcinoma on the part of the acid-secreting as compared with the antral mucosa. The early loss of methylcholanthrene from the antral site is probably due to its evacuation into the lumen of the stomach as a result of the early development of the adenomatous diverticulum as described. The possible rôle of other factors influencing this phenomenon requires study. However, there is a provocative analogy between man and the rat in that in both the antral mucosa is more susceptible to the development of epithelial neoplasms than the acid-secreting mucosa. The factors accounting for this phenomenon are as yet undemonstrated.

SUMMARY AND CONCLUSIONS

A dose of 0.6 mg. of 20-methylcholanthrene was injected intramurally into each of the two sites of the glandular stomach of rats of 3 strains. The first injection site was at the antrum, near the pylorus. The second injection site was at the acid-secreting portion of the glandular chamber, near the limiting ridge. A total of 265 rats of 3 inbred strains came to autopsy. The most frequent lesion induced was an adenomatous diverticulum, which occurred at the first or at the second or at both injection sites 273 times in 220 rats. It is regarded as precancerous. Many of the adenomatous diverticula showed squamous-cell metaplasia of the glandu-

lar mucosa and metaplastic bone formation in the stroma. There were 28 malignant tumors in 26 animals as follows: 4 adenocarcinoma, 4 adenoacanthoma, 3 mixed carcinoma and sarcoma, 16 sarcoma, and 1 unclassified malignant tumor. Paralleling the experience in man, in whom the antral region is a frequent site for gastric carcinoma, most of the benign and malignant epithelial tumors in the rat also arose in this location. Of the 19 sarcomas, or mixed sarcoma and carcinoma, 6 occurred at the antral site, 11 at the acid-secreting site, and 2 embraced both sites. The mesenchymal growths therefore exhibited a slight predilection for the acid-secreting portion of the glandular stomach in direct contrast to the epithelial tumors. In 19 cases there was no neoplastic response at either injection site.

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PLATE 92

FIGURE 1.—External view of stomach removed immediately after operation, showing the two sites of injection in wall of the glandular stomach. The first site is in the antrum; the second in the acid-secreting portion. Duodenum to left, esophagus top center, limiting ridge extending downward from esophagus, glandular stomach to left, forestomach to right. × 1

FIGURE 2.—Stomach. In the antrum (first injection site) there is an umbilicated lesion; second site of injection at acid-secreting portion (arrow) shows small scar. Duodenun above, forestomach to left. × 2

FIGURES 3 and 4.—Stomach. External (3) and internal (4) view. Adenomatous diverticulum, solitary cystic type, first injection site. Second injection site not visible. × 1

FIGURE 5.—Stomach showing two adenomatous diverticula (one at each injection site); combined cystic and solid types. × 125

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PLATE 93

FIGURES 6 and 7.—Stomach showing combined cystic and solid type adenomatous diverticulum; first site of injection. Figure 6 external view; figure 7, cut surface. Circa × 2

FIGURES 8 and 9.—Stomach showing combined cystic and solid adenomatous diverticulum at first injection site. Figure 8, external view; figure 9, cut surface. × 1

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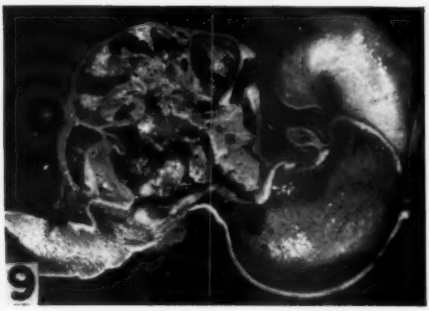
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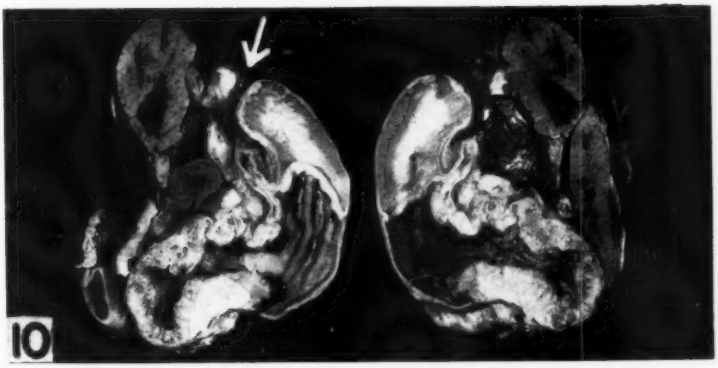
PLATE 94

FIGURE 10.—Hemisections of stomach. Carcinoma, first injection site, involving lesser curvature and antrum. Between fundus of forestomach and liver is an enlarged lymph node (arrow) containing a metastasis. × 1

FIGURE 11.—Stomach. Compressed forestomach left upper part of illustration, limiting ridge marked by arrow. A portion of wall of glandular stomach at first injection site replaced by carcinomatous tissue which invades liver. Second site of injection (not shown in the illustration) exhibited no neoplastic response. Circa × 2

FIGURE 12.—Forestomach, esophagus, limiting ridge upper right; duodenum left; glandular stomach to right of duodenum. The wall of the lesser curvature and antral portion of greater curvature of glandular chamber thickened by sarcoma. The dark band along the lesser curvature is a remnant of the muscularis propria. Circa × 2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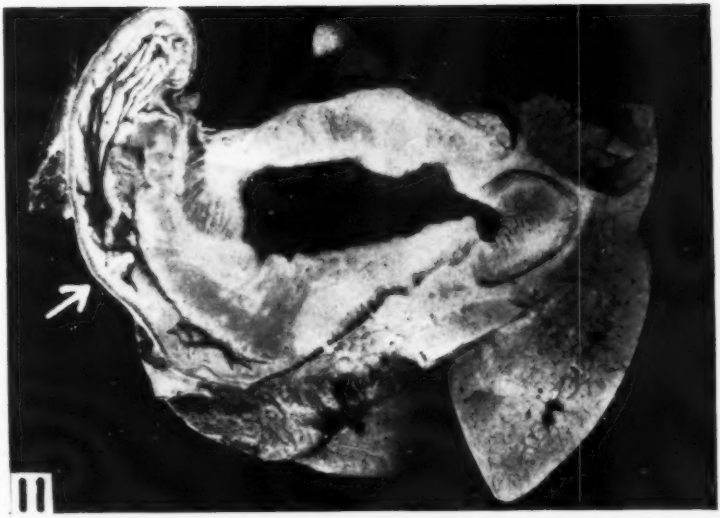
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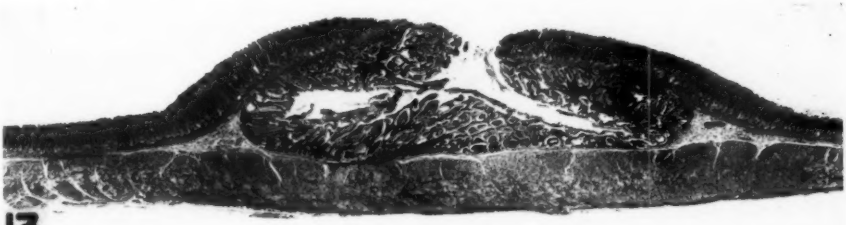
PLATE 95

FIGURE 13.—Section through umbilicated adenomatous diverticulum corresponding to stage of development shown in figure 2. Atypical glandular mucosa which lines the defect is composed exclusively, or nearly so, of mucous type cells and has extended through muscularis mucosa into the submucosa. The lesion, although dimpled, is not ulcerated. There is relatively little inflammatory reaction. × 15

FIGURE 14.—Section through junction of normal stomach (above) and large adenomatous diverticulum, which shows both cystic and solid areas. Small dark areas are focal collections of inflammatory cells. Solid gray areas are fibrous tissue. The entire lesion is encapsulated peripherally even though some glands are relatively deep to the mucosal surface. × 10

FIGURE 15.—Area from adenomatous diverticulum showing: characteristics of glands, excessive mucus production, hyperchromatism of cells and relationship between rather aggressive glands and muscle (on right), despite which the serosa was not infiltrated. × 40



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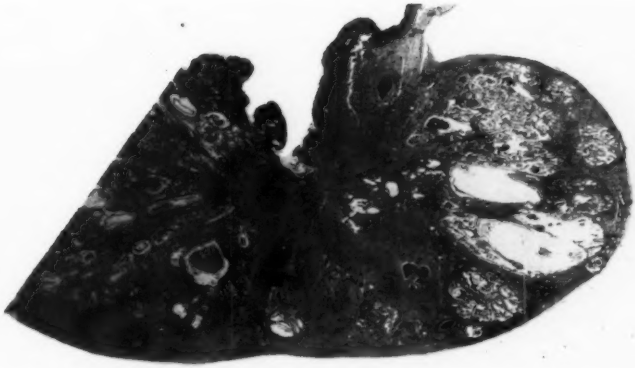

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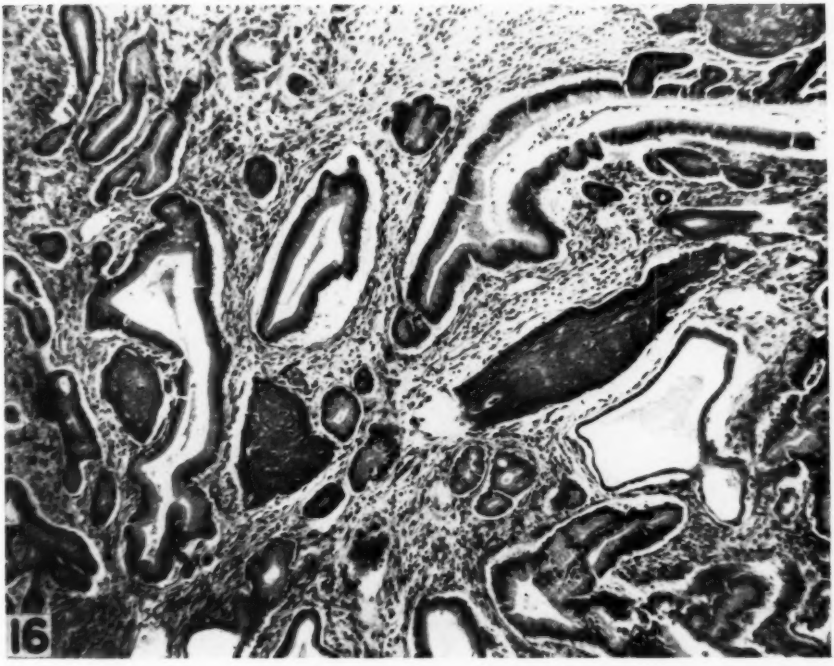
PLATE 96

FIGURE 16.—Area from adenomatous diverticulum showing appearance of glands and metaplastic bone in stroma. × 90

FIGURE 17.—Area from adenomatous diverticulum; severe inflammatory reaction involving glands and stroma. The inflammatory cells are chiefly plasma cells and lymphocytes. × 240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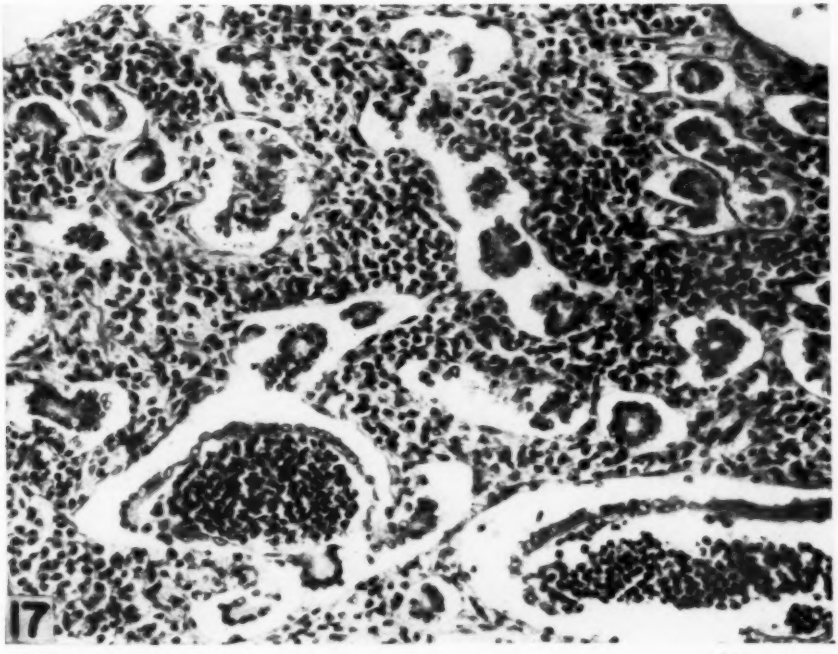
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PLATE 97

FIGURE 18.—Section from periphery of adenomatous diverticulum, showing encapsulation at serosal surface, sharply delimiting the proliferating glands. Smooth muscle elements are frequently identified in this peripheral capsule, indicating that although attenuated, the wall of stomach is intact. The epithelial cells lining the deepest aspect of some of the glands are atrophic or degenerated. × 160

FIGURE 19.—Area from mucosal surface of adenomatous diverticulum located at the antrum (not in continuity with the forestomach mucosa) showing squamous metaplasia of the surface and superficial glands; deeper glands here are lined by mucous cells. Note edema and inflammatory cells of stroma. × 120

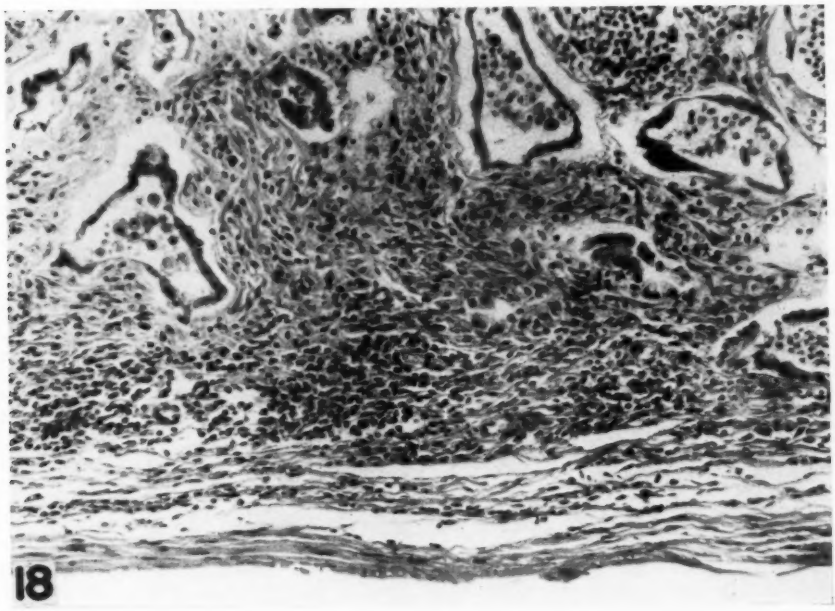
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PLATE 98

Adenomatous diverticulum, first site. All three illustrations from same specimen showing solid and cystic polypoid areas, with inflammation of stroma and glands.

FIGURE 20.—Antrum (duodenum on left, normal stomach on right). Abrupt penetration through muscularis propria (below) with resultant cyst formation on serosal surface; the cyst wall appears incomplete because a piece of it was excised for transplantation. Although showing aggressive features, and considered a borderline case, this tumor was not classified in the carcinoma group. × 10

FIGURE 21.—Transition from normal stomach (right) through hyperplastic junctional area to adenomatous diverticulum (left). Below there is thinning of muscularis propria, and the wall of the stomach here is composed chiefly of subserosal inflammatory and scar tissue. × 15

FIGURE 22.—Higher power view of area showing penetration of muscularis by cystic structure shown in figure 20, but a few histologic sections removed from it. Blunt margin of muscle on left suggests pressure break rather than true infiltration. × 40

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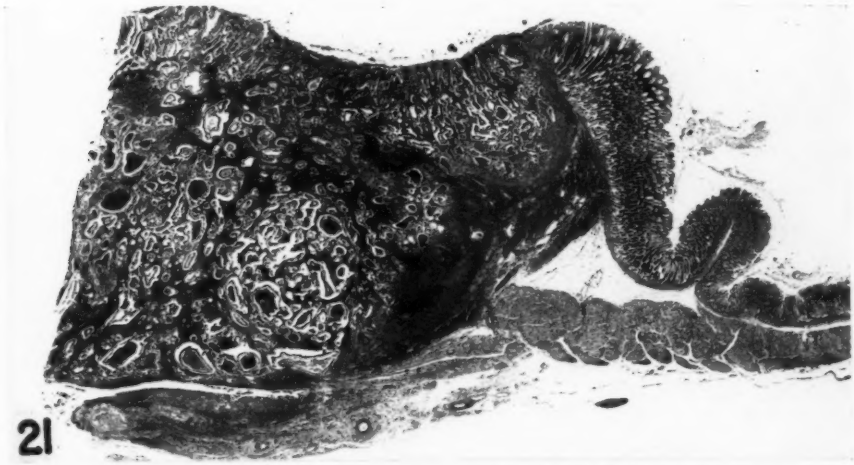
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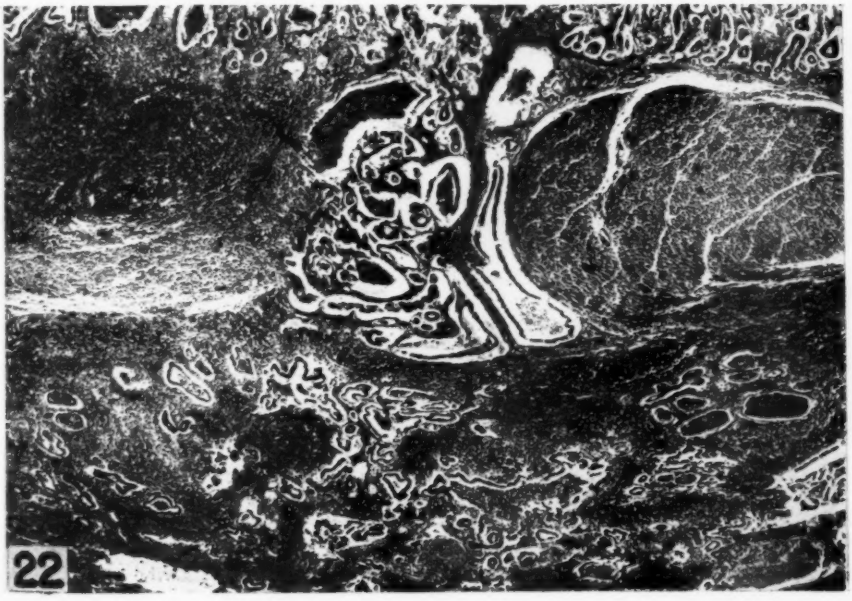
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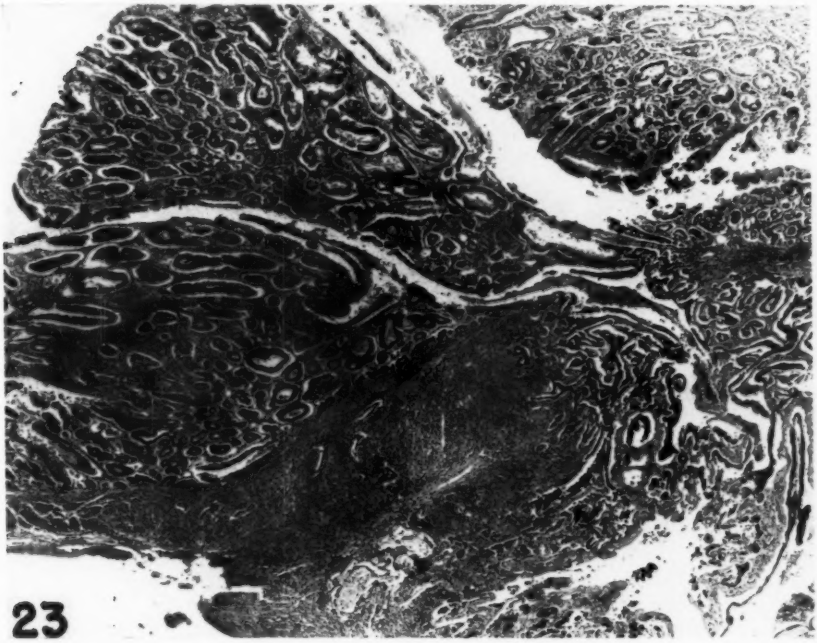
PLATE 99

FIGURE 23.—Adenomatous diverticulum—a yet more aggressive type lesion than that shown in figures 20, 21, and 22. Note individual glands within the muscularis propria (lower left part of illustration). The serosa, however, was intact. The lower right portion of the lesion is a part of a large adenomatous diverticulum which was everywhere encapsulated (not shown in illustration). Note sinus tract which begins at left margin of illustration, extends from left to right, and terminates in cystic area (lower right). Such sinuses facilitate the introduction of infection from the gastric lumen (left border) into the depth of the lesion. This and the specimen in figure 20 are borderline lesions which we believe illustrate transitions between the adenomatous diverticulum and carcinoma. These two lesions might with justification be included in either category. Because of our conservative viewpoint of not making a diagnosis of experimental gastric carcinoma in the absence of penetration of the serosa and the establishment of an obvious infiltrating neoplastic deposit thereon, the two lesions are coded as adenomatous diverticulum. × 40

FIGURE 24.—Gastric adenocarcinoma infiltrating pancreas. This is a section of the lesion illustrated in figure 11. × 200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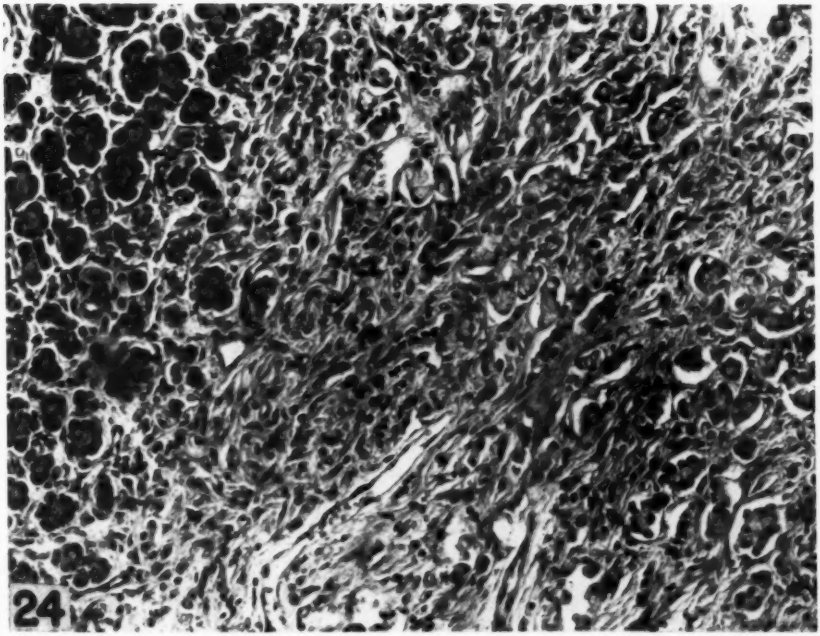
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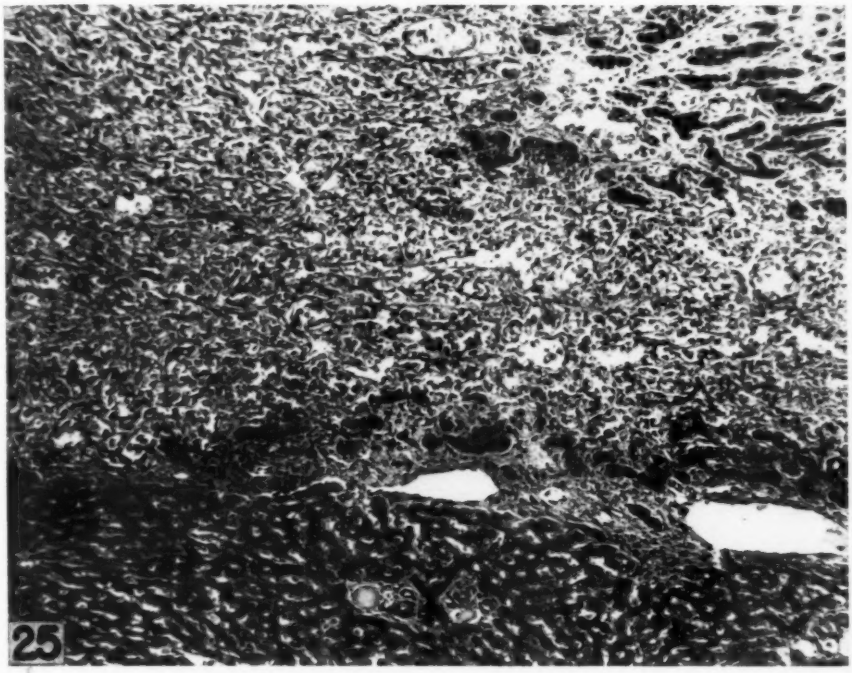
PLATE 100

FIGURE 25.—Gastric adenocarcinoma infiltrating liver. This is a section of the lesion illustrated in figure 11. × 120

FIGURE 26.—Stomach showing adenomatous diverticulum (left) and adenocarcinoma (right) which permeates lymphatic vessels (arrow) extends onto the serosa and into mesentery and has metastasized to pancreas (rectangle). The gross appearance of this lesion is illustrated in figure 10. × 6



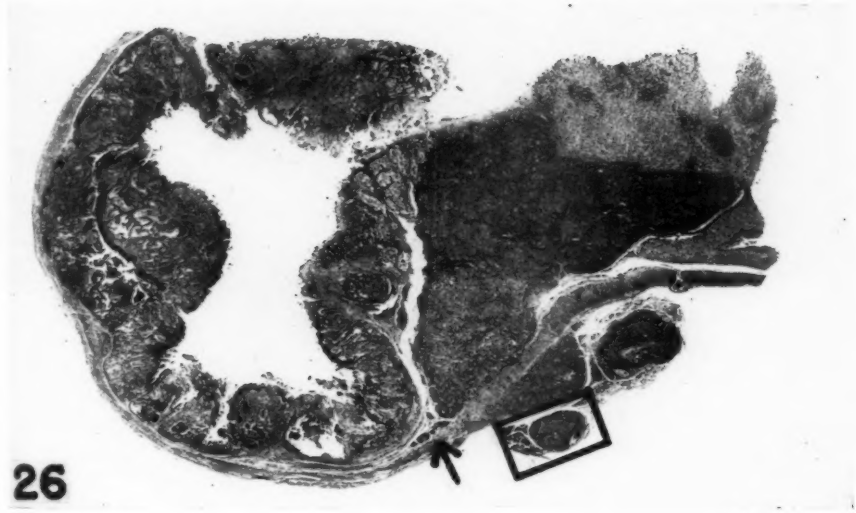


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PLATE 101

FIGURE 27.—Area within rectangle of figure 26 showing circumscribed deposit of carcinoma in pancreas and carcinomatous permeation of lymphatic vessels to left. × 60

FIGURE 28.—Lymph node. Metastatic carcinoma (see arrow figure 10, not, however, shown in figure 26 although from a different portion of the same specimen). × 250

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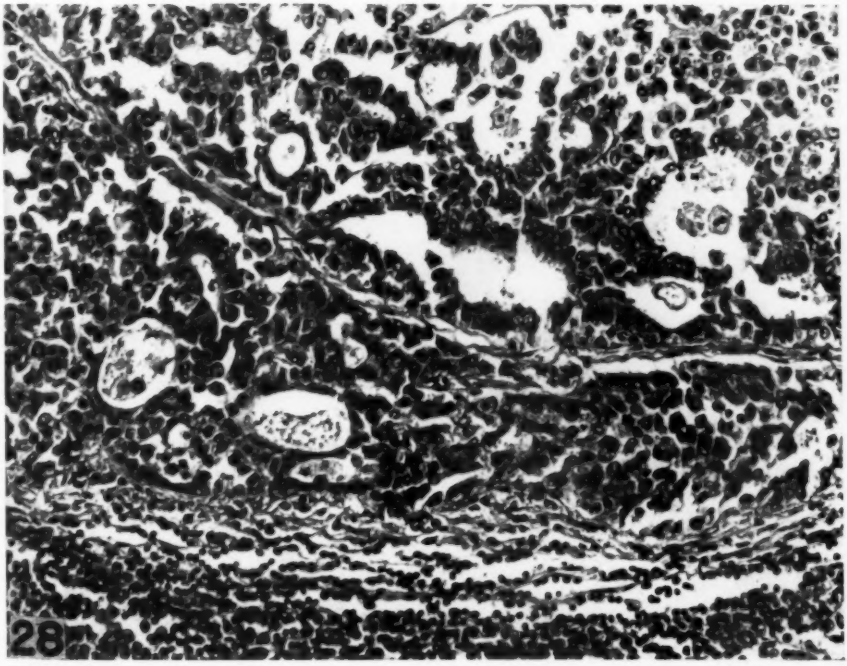
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PLATE 102

FIGURE 29.—Stomach (same specimen illustrated in figure 10 and figure 26), showing carcinoma infiltrating muscularis propria and forming serosal nodule which extends into mesentery. × 40

FIGURE 30.—Area from adenomatous diverticulum. Nodule of atypical, aggressive, proliferating squamous epithelium, histologically consistent with squamous carcinoma. The adenomatous diverticulum, however, was well delimited with the peripheral margins everywhere surrounded by a wall of fibrous tissue. The nodule illustrated is probably an early developing stage of squamous carcinoma. It is conceivable that if transplanted to other rats it might produce a carcinoma at site of inoculation; for discussion of this problem see text dealing with transplantation. × 100

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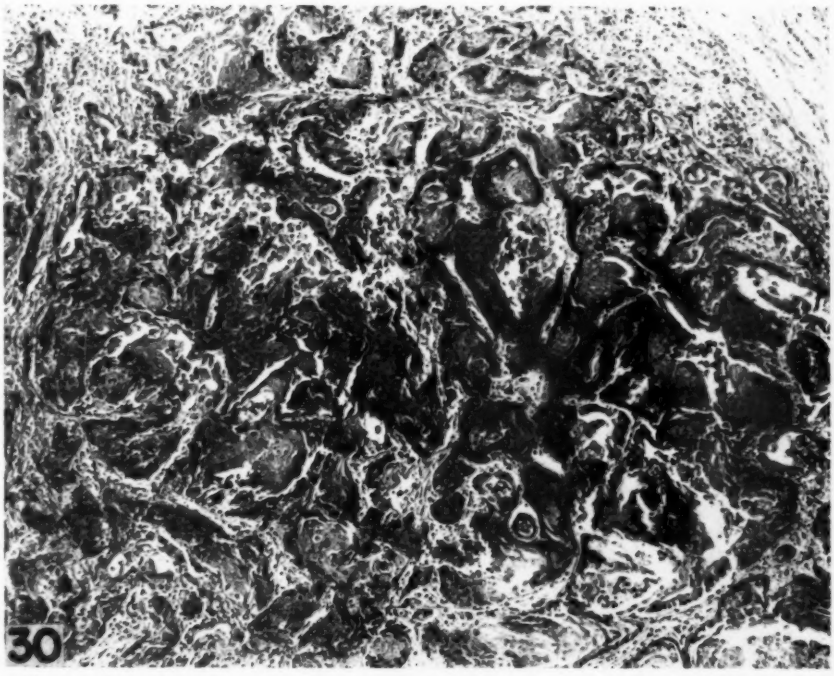

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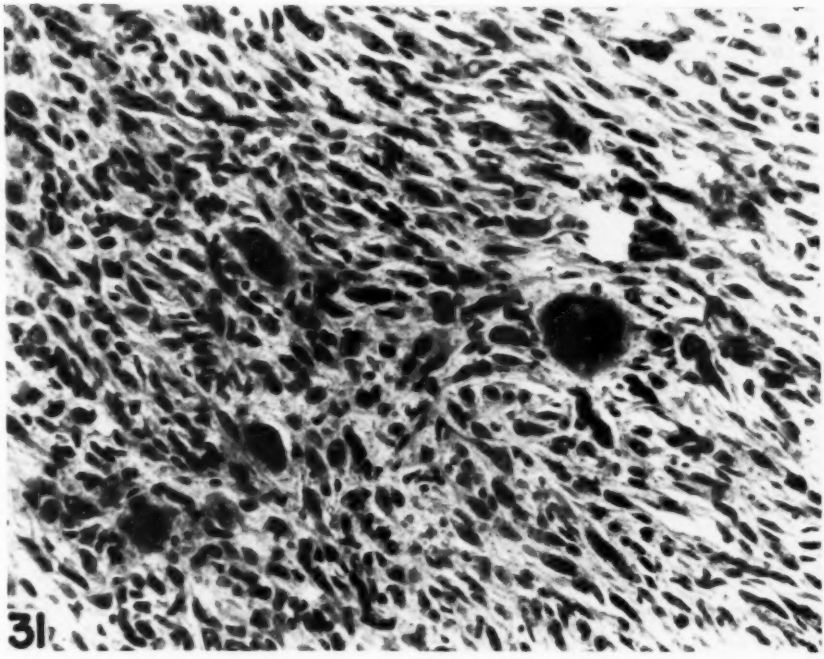
PLATE 103

FIGURE 31.—Stomach—sarcoma. × 125

FIGURE 32.—Stomach—osteogenic sarcoma. × 240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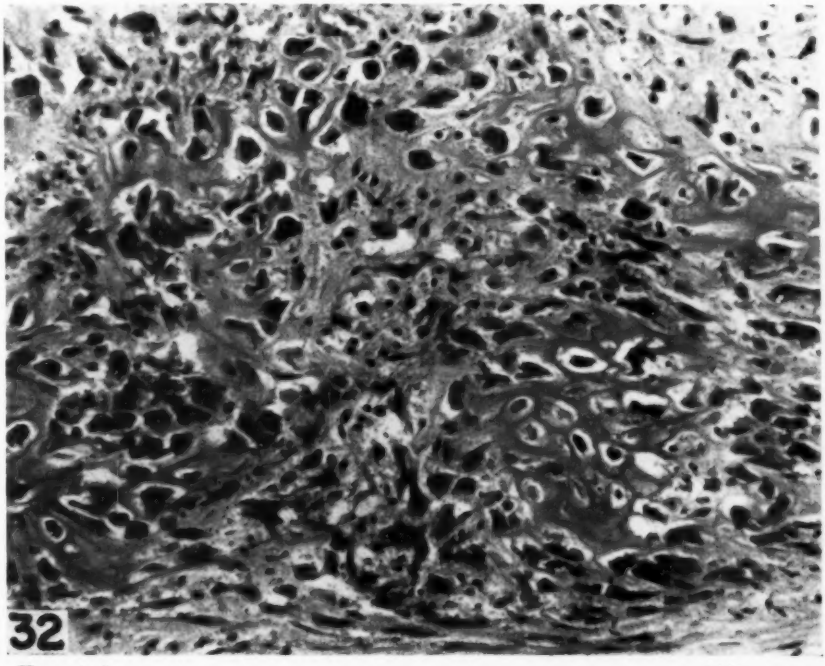
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THE PRODUCTION OF SARCOMAS FROM CULTURED TISSUES OF HEPATOMA, MELANOMA, AND THYROID TUMORS[1]

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Tissues from a hepatoma of the mouse, the Cloudman melanoma, and a tumor of thyroid parenchyma were cultured *in vitro* to isolate the primary cells of the three tumors. Cultures were injected periodically into mice to test the ability of the cultured cells to give rise to tumors. The results of these injections are summarized in the present study.

MATERIALS AND METHODS

SOURCES OF TISSUE

Hepatoma tissue.—This tumor arose in the liver of a 17-month-old male mouse belonging to the Andervont line of strain C3H (*1*). Tissue cultures were planted from tumor tissue that had been carried *in vivo* for 47 generations.[3] The tissue used for culture was taken from a 4½-month-old mouse 6 weeks after implantation.

Thyroid tumor tissue.—This tumor of thyroid parenchyma originated in a strain C3H mouse that had been fed thiouracil and 0.05 percent 2-acetylaminofluorene for a period of 16 months (*2*).[4] One series of tissue cultures, series 1,252, was planted from tumor tissue that had been carried *in vivo* for six generations in the strain C3H mouse. This particular tumor line (*3*) was one that had been found to have some functional activity *in vivo*. A second series of tissue cultures, series 1,251, was planted from tissue that had been carried for 9 generations *in vivo*.

Melanoma tissue.—Tissue from the Cloudman melanoma S91 was used to plant three series of tissue cultures.[5] Series 400 and 402 were planted from tumor tissue that had been carried for 27 and 28 generations, respectively, in strain DBA mice. Series 401 was planted from amelanotic melanoma tissue that had been carried for 27 generations in strain C mice (*4*).

[1] Received for publication January 14, 1952.
[2] National Institutes of Health, Public Health Service, Federal Security Agency.
[3] Obtained from Dr. A. J. Dalton of this Institute.
[4] Obtained from Dr. H. P. Morris of this Institute.
[5] Obtained from Dr. G. H. Algire of this Institute.

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Culture Procedures

The tumor tissue was dissected and cut into explants approximately $1 \times 2 \times 8$ mm. Representative strips of the tissue were also fixed in formalin-Zenker, sectioned, and stained with hematoxylin and eosin and van Gieson's picro-fuchsin. The explants of the hepatoma and melanoma were planted under perforated cellophane in D3.5 Carrel flasks (*5*, *6*); the tissues of the thyroid tumor were planted in hen plasma clot in D3.5 Carrel flasks (*7*). The supernatant fluid medium of the cultures consisted of 1 ml. of a mixture of 40 percent horse serum, 20 percent chick-embryo extract, and 40 percent balanced saline. This fluid was renewed three times a week.

The method of cell transplantation used for the plasma substrate cultures has been described (*7*). In a few of the cultures the cells lysed the plasma clot, but the cells of these cultures continued to proliferate on the glass floor of the Carrel flask. These cells growing on glass substrate were scraped from the floor of the flask by means of a platinum-iridium spatula (*7*), and the cell suspension from each flask was transferred by pipette to two sterile flasks. This transfer procedure was used at each transplantation. A few cultures of thyroid tumor tissue were thus carried on a glass substrate from the fourth transplant generation to the end of the study.

The method of cell transplantation used for the cellophane substrate cultures consisted in scraping the cells from the cellophane substrate and from the floor of the flask by means of a platinum-iridium spatula. The cell suspension was then transferred by pipette to one or two sterile flasks, each containing a sheet of perforated cellophane. The original culture was often retained, and the cells were scraped from the substrate at later dates to prepare other daughter cultures. Thus, daughter cultures from 1 original culture were not always prepared on the same date.

At the third transplant generation, cultures of the hepatoma were transferred to the T-60 type culture flask,[6] and all subsequent hepatoma cultures were grown in this type of flask. The culture medium consisted of a cellophane substrate and a volume of 5 to 10 ml. of supernatant culture fluid.

Injection of Cultures Into Mice

At intervals after planting, cultures of the hepatoma and thyroid tumor were injected intramuscularly into strain C3H male mice of approximately 1 to 4 months of age; cultures of the melanoma were injected intramuscularly into strain DBA male mice. The injection procedures for the cultures of melanoma and thyroid tumor tissue have been described (*8*, *9*). Cells of the hepatoma cultures were scraped from the cellophane substrate of the T-60 flask in a 10 ml.-volume of culture fluid. Each cell suspension was then centrifuged at 1,100 r. p. m. for 5 minutes.

[6] T-60 flask design to be published.

The supernatant fluid was discarded, and the pellet of cells was suspended in a volume of 0.5 ml. of culture fluid and injected. Cells were injected from the cultures at varying intervals after transplantation when a dense growth of cells was observed in the flasks.

The mice were kept under observation for 3 to 12 months after injection. Autopsies were performed on all mice. Strips of tumor tissue were fixed in formalin-Zenker, sectioned, and stained with hematoxylin and eosin, and van Gieson's picro-fuchsin.

Cell Observations and Photographic Records

The cultures were examined microscopically for changes in cell condition or morphology. Photographic records were made at intervals by means of the photomicrographic methods previously described (*10*, *11*), except that the cultures were photographed at varying ages.

RESULTS

Injections of Hepatoma Tissue Cultures

The results of injecting cells from cultured hepatoma tissue are presented in a condensed genealogy (text-fig. 1) that shows the relationship of all tissue cultures injected, the main lines of descent from which these cultures arose, and the distribution of the injected cultures with respect to transplant generation. Of a total of 91 cultures injected, 26 gave rise to tumors. One of these tumors was palpable 13 days after injection of the tissue culture. The tumors on microscopic examination were diagnosed as fibrosarcomas.[7]

All of the cultures in this series arose from 1 culture in the third transplant generation (text-fig. 1). As may be noted in the genealogy chart, the cultures descended from this one culture varied markedly in their capacity to produce sarcomas when injected into mice. Even cells injected at different times from the same culture (text-fig. 1) varied in their capacity to produce sarcomas. The first cultures to give rise to sarcomas were in the fourth transplant generation (text-fig. 1), and were injected about 5 months after explanting of the hepatoma tissue from the mouse. Since all cultures of a transplant generation were not planted on the same date, and since all cultures of a transplant generation were not injected on the same date, it was impossible to indicate dates of transplantation or injection in the condensed genealogy (text-fig. 1). The dates of injection are, therefore, separately presented in text-figure 2.

The morphologic patterns of the original hepatoma tissue used for planting the cultures, of a representative tissue culture, and of a sarcoma produced by injecting a tissue culture into a strain C3H mouse are presented in figures 1 to 3.

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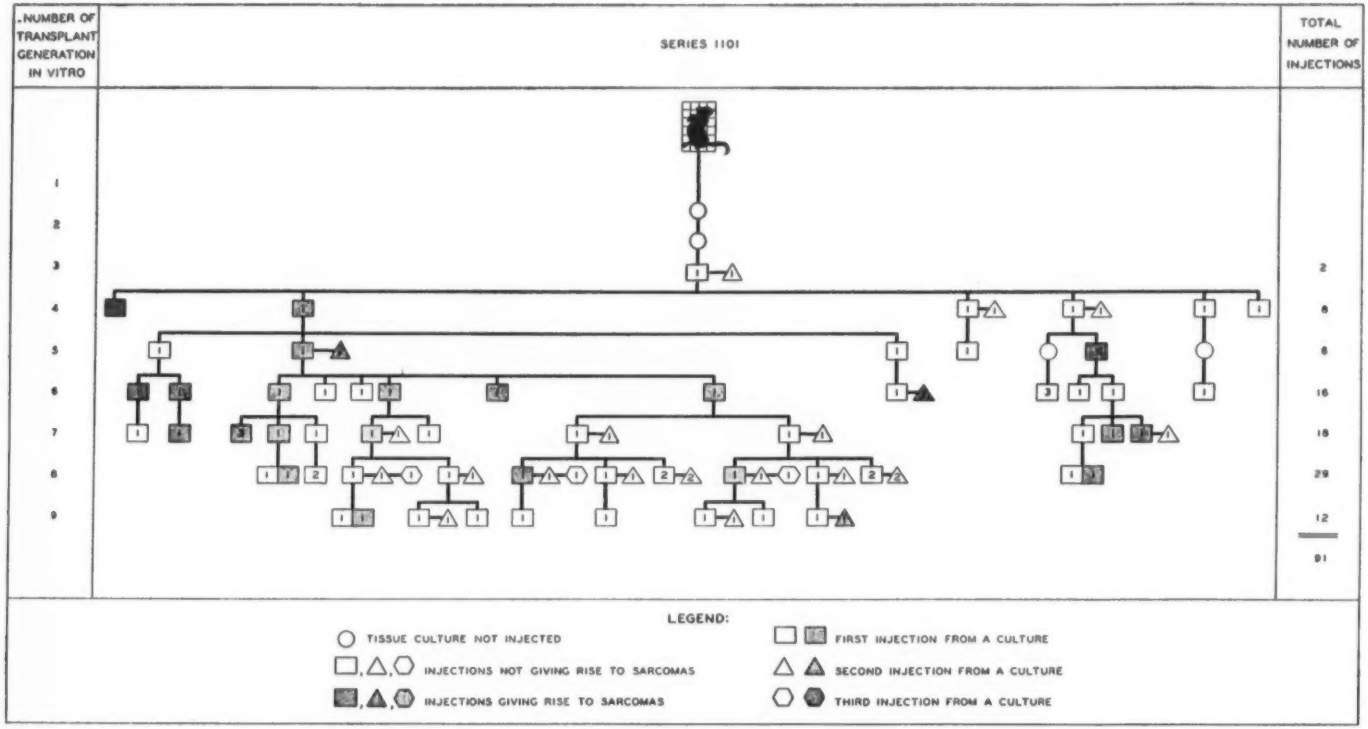
[7] The diagnosis of all tumor sections was confirmed by Dr. Thelma B. Dunn of this Institute, whose assistance is gratefully acknowledged.



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Injections of Thyroid Tumor Tissue Cultures

The results of injecting cultures of thyroid tumor tissue of series 1,251 are summarized in a condensed genealogy (text-fig. 3) that shows the origin, relationships, and transplant generation of all cultures injected. Sixty-one tissue cultures were injected into strain C3H mice, and 26 of these gave rise to tumors. Some of these tumors were palpable 18 to 20 days after injection of the tissue culture. All tumors were diagnosed on microscopic examination as sarcomas. Four of these sarcomas were subinoculated and grew in all 16 mice inoculated.

The sarcoma-producing cultures arose from 3 different explants of the original thyroid tumor tissue (text-fig. 3). The first culture to give rise to a sarcoma was in the fourth transplant generation (text-fig. 3). This culture was injected about 4 months after explanting of the thyroid tumor tissue from the mouse (text-fig. 2).

The histologic appearance of the original tissue used to plant the cultures, the morphology of a representative tissue culture, and the pattern of the sarcoma produced by injecting this tissue culture are illustrated in figures 4 to 6.

The results of injecting cultures of thyroid tumor tissue of series 1,252 are summarized in text-figure 4. A total of 104 cultures were injected, and 19 gave rise to tumors. Some of these tumors were palpable 12 to 18 days after injection of the tissue culture. On microscopic examination, all tumors were diagnosed as sarcomas. One of these sarcomas was subinoculated and grew in all 6 mice inoculated. Sixteen of the cultures injected were grown on glass substrate, rather than in plasma substrate; none of these cultures produced a sarcoma.

Text-figure 1.—Condensed genealogy of injected cultures of mouse hepatoma tissue, series 1,101. The number of transplant generation is indicated in the column at the left. The column at the right gives the number of injections made during each transplant generation. Each circle represents a tissue culture that was not injected. When cells were scraped from the cellophane substrate and from the floor of the T-60 flask for the planting of daughter cultures or for injection, some cells remained on the substrate. The original flask culture was therefore often maintained, and cells were scraped from the substrate at later dates and used for planting additional daughter cultures or for additional injections.

The first injection of cells from a culture is indicated by a rectangle. The second injection of cells from the culture is indicated by a triangle, and a third injection of cells from the same culture is indicated by a hexagon. The number in the rectangle, triangle, or hexagon indicates the number of injections made. Shaded rectangles, triangles, or hexagons indicate that sarcomas arose from the injected cells.

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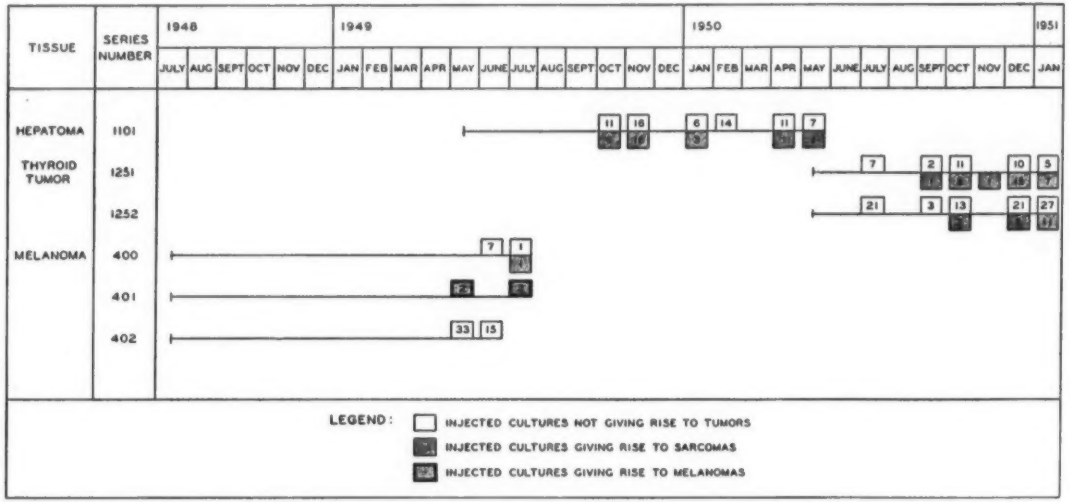
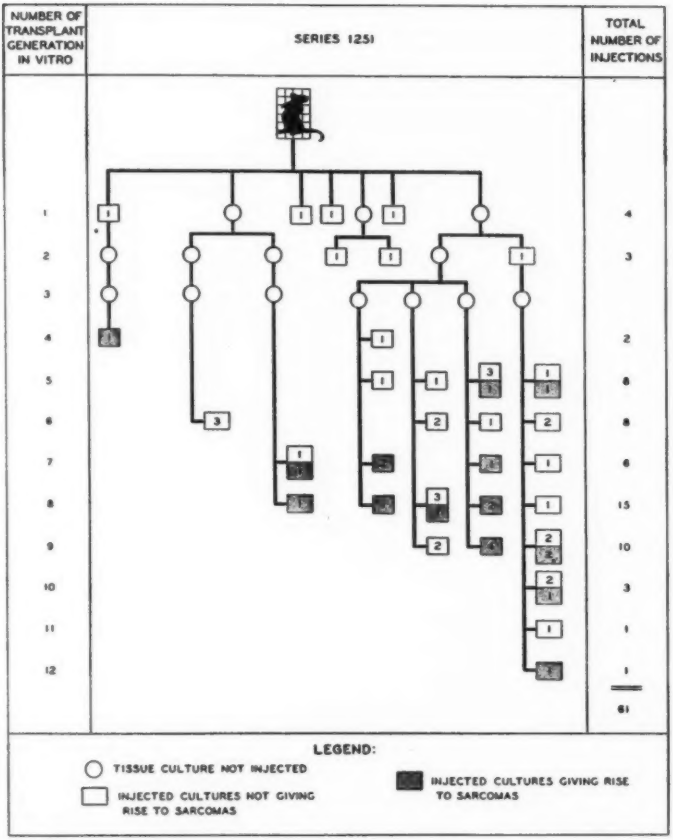
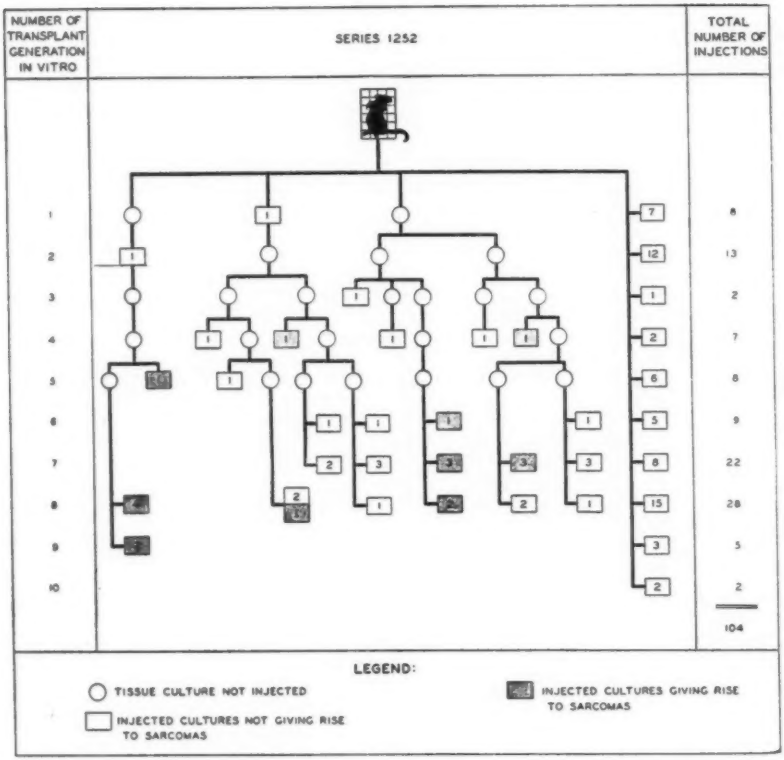


TEXT-FIGURE 2.—Chronologic relationship of the cell strains studied. The tumor tissues explanted and series numbers are shown at the left. The horizontal lines to the right of these columns indicate the period of culture of the cell strains studied. The rectangles represent groups of injections made from cultures at the times indicated. The number in each rectangle indicates the number of injections, each into a different mouse. The shaded rectangles indicate groups of injections that gave rise to sarcomas. The heavily outlined, shaded rectangles indicate groups of injections that gave rise to melanomas.



TEXT-FIGURE 3.—Condensed genealogy of injected cultures of thyroid tumor tissue, series 1,251. The number of transplant generation is indicated in the column at the left. The column at the right gives the number of cultures injected at the close of each transplant generation. Each circle represents a tissue culture that was not injected. Each rectangle represents one or more cultures injected into mice. The number in the rectangle indicates the number of cultures injected, each into a different mouse. The shaded rectangles indicate groups of cultures that gave rise to sarcomas.

The complete culture genealogy is shown through the third transplant generation. When each culture of the first and second transplant generation was injected, a small section of the plasma substrate culture, approximately $1 \times 5 \times 15$ mm. in size was retained and immediately used to plant a culture of the next generation. In this way culture lines were continued. Two culture lines, shown at the extreme left and extreme right of the chart, originated from such injected cultures of the first and second transplant generations, respectively. From the fourth generation on, only those cultures that were actually injected are shown.



TEXT-FIGURE 4.—Condensed genealogy of injected cultures of thyroid tumor tissue, series 1,252. The number of transplant generation is indicated in the column at the left. The column at the right gives the number of cultures injected at the close of each transplant generation. Each circle represents a tissue culture that was not injected. Each rectangle represents one or more cultures injected into mice. The number in the rectangle indicates the number of cultures injected, each into a different mouse. The shaded rectangles indicate groups of cultures that gave rise to sarcomas.

The genealogy of the three culture lines that gave rise to sarcoma-producing cultures is shown in detail. Injections from all other culture lines are summarized and tabulated according to transplant generation on the vertical line at the right of the chart.

The complete culture genealogy of the three culture lines is shown through the fifth transplant generation. When each culture of the first and second transplant generation was injected, a small section of the plasma substrate culture, approximately $1 \times 5 \times 15$ mm. in size, was retained and immediately used to plant a culture of the next generation. In this way culture lines were continued. The first two culture lines on the left originated from such injected cultures of the second and first generations, respectively. From the sixth generation on, only those cultures that were actually injected are shown.

Twenty-two cultures were planted from the tumor tissue excised from the mouse; 14 of these cultures were not carried for longer than the first 3 generations *in vitro;* of the 8 culture lines carried for longer than 3 generations, 3 gave rise to sarcoma-producing cultures. The sarcoma-producing cultures thus arose from three different explants of the tumor tissue (text-fig. 4). The first culture to give rise to a sarcoma was in the fourth transplant generation (text-fig. 4). This culture was injected approximately 5 months after explanting of the thyroid tumor tissue from the mouse (text-fig. 2).

Photomicrographs of a section of the original tissue used to plant the cultures, of a representative tissue culture, and of a section of the sarcoma produced by injecting this tissue culture are presented in figures 7 to 9.

Injections of Melanoma Tissue Cultures

Two series of tissue cultures, series 400 and 402, were planted, each from a Cloudman S91 melanoma. One series of tissue cultures, series 401, was planted from an amelanotic melanoma tumor. The results of injecting cultures of these three series into strain DBA mice are summarized in text-figure 5. Thirteen cultures of series 400 were injected. One tumor was produced by the injection of 3 cultures into 1 mouse. The cultures that gave rise to this tumor were injected about 13 months after explanting of the melanoma tissue from the mouse (text-fig. 2). On microscopic observation, no pigment was observed in the cells of this tumor. The tumor was, therefore, diagnosed as a sarcoma or as an amelanotic melanoma.

The histologic pattern of the original S91 melanoma tissue used to plant the cultures, the morphology of the cells in culture, and the histologic pattern of the sarcoma or amelanotic melanoma are illustrated in figures 10 to 12.

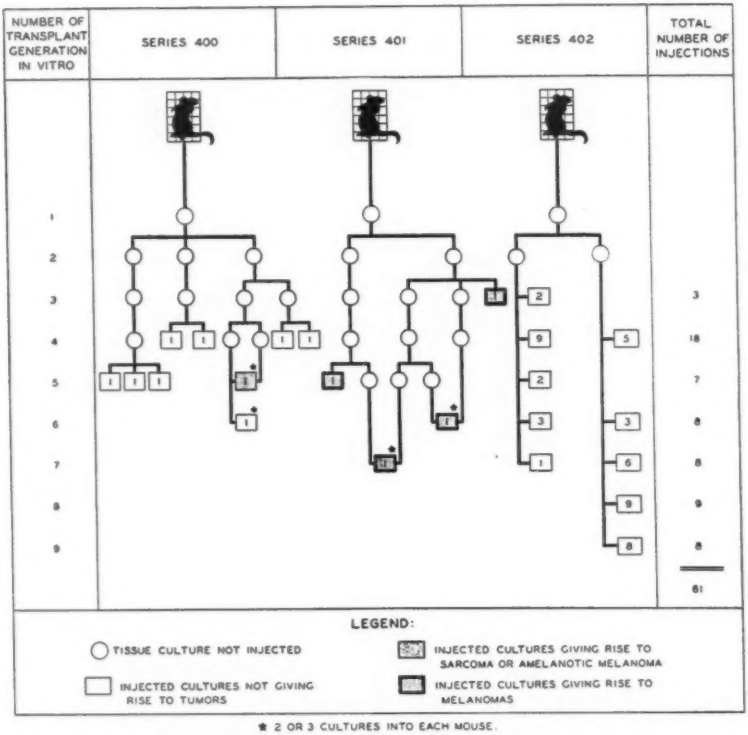
All cultures of series 401 that were injected gave rise to melanotic melanomas in strain DBA mice. One of these melanomas was subinoculated and grew as a heavily pigmented melanoma in all 4 mice inoculated.

Photomicrographs of the original tissue used to plant series 401, of the tissue culture cells, and of the melanoma produced by injection of a culture are presented in figures 13 to 15.

Forty-eight cultures of series 402 were injected. No tumors were produced.

DISCUSSION

There are several possible interpretations of the production of sarcomas in mice by injection of cultured tissues of hepatoma, melanoma, and thyroid tumors. One of the less plausible concepts is that the original tumors used to plant the cultures may have been mixed tumors, containing sarcoma cells in addition to the primary tumor cells. This interpretation may be plausible for the Cloudman melanoma. A strain of completely amelanotic sarcoma-like cells may have been selectively cultured from



TEXT-FIGURE 5.—Condensed genealogy of injected cultures of series 400, 401, and 402. Cultures of series 400 and 402 originated from Cloudman S91 melanomas. Cultures of series 401 originated from an amelanotic melanoma.

The number of transplant generation is indicated in the column at the left. The column at the right gives the number of cultures injected at the close of each transplant generation. Each circle represents a tissue culture that was not injected. Each rectangle represents one or more cultures injected into mice. The number in the rectangle indicates the number of cultures injected, each into a different mouse, except in four cases where two or three cultures were injected into one mouse. Each of these four injections is indicated by an asterisk. A shaded rectangle indicates that the injection gave rise to a sarcoma. A heavily outlined shaded rectangle indicates that the injection gave rise to a melanoma.

In series 400, the complete genealogy is shown through the fourth transplant generation. From the fifth generation on, only those cultures that were actually injected are shown.

In series 401, the complete genealogy is shown through the fifth transplant generation. From the sixth generation on, only those cultures that were actually injected are shown.

In series 402, the complete genealogy is shown only through the second transplant generation. From the third generation on, only those cultures that were actually injected are shown.

the melanoma tissue, but studies of both the tumor of the thyroid parenchyma and of the hepatoma have revealed no evidence of sarcomatous change in the stroma of either of these tumors.[8]

A second interpretation is that the primary tumor cell, which appeared to be characteristic of the original tumor tissue, underwent an alteration in morphology in culture so that when reinjected intramuscularly into mice the tumors consequently showed a different morphologic design. From the results of subinoculations, this modified sarcoma-like tissue pattern persisted through at least two passages *in vivo*.

A third interpretation is that the primary tumor-cell type was overgrown in culture by fibroblasts. In support of this interpretation is the fact that all cultured cells resembled fibroblasts in morphology. It is known that fibroblasts cultured from normal subcutaneous connective tissue or skeletal muscle tissue of the strain C3H mouse transform under the culture conditions used in the present study, and become able to give rise to sarcomas when injected into mice (*9*). In the present study, the sarcomas produced may thus have arisen from such altered fibroblasts.

It is impossible at the present time to know which, if any, of these interpretations is correct.

SUMMARY

Tissues from a hepatoma of the mouse, the Cloudman melanoma, and a tumor of thyroid parenchyma of the mouse were cultured *in vitro* to isolate the primary tumor cells. The hepatoma arose spontaneously in the liver of a strain C3H mouse and had been carried *in vivo* for 47 generations. The tumor of the thyroid parenchyma originated in a strain C3H mouse that had been fed thiouracil and 0.05 percent 2-acetylaminofluorene for a period of 16 months. The two tumors used had been carried *in vivo* for 6 and 9 generations. The melanoma tissue was from the Cloudman melanoma S91. Both amelanotic melanoma and melanotic tumors were used that had been carried *in vivo* for 27 and 28 generations in strains C and DBA mice, respectively.

Cultures of the hepatoma and thyroid tumor were injected periodically into C3H strain mice to test the ability of the cultured cells to give rise to hepatomas and thyroid tumors, respectively. Cultures of the Cloudman melanoma were injected into strain DBA mice. Cultures of both hepatoma and thyroid tumor produced a high incidence of tumors when injected, but all tumors were diagnosed as sarcomas. The first cultures to produce tumors were injected approximately 5 months after explanting of the tumor tissues from the mouse. Three cultures of melanoma S91 tissue injected into one mouse 13 months after explanting produced a sarcoma or amelanotic melanoma.

[8] This information was obtained from Dr. A. J. Dalton of this Institute, who has made extensive studies of the histology of these two tumors.

Three possible interpretations of the production of sarcomas in mice by the injection of cultured tissue of hepatoma, melanoma, and thyroid tumors have been suggested:

1) the original tumors used to plant the cultures may have been mixed tumors containing sarcoma cells and primary tumor cells;
2) the primary tumor-cell type underwent an alteration in morphology in culture; and
3) the primary tumor-cell type was overgrown in culture by fibroblasts that transformed to sarcoma-producing cells.

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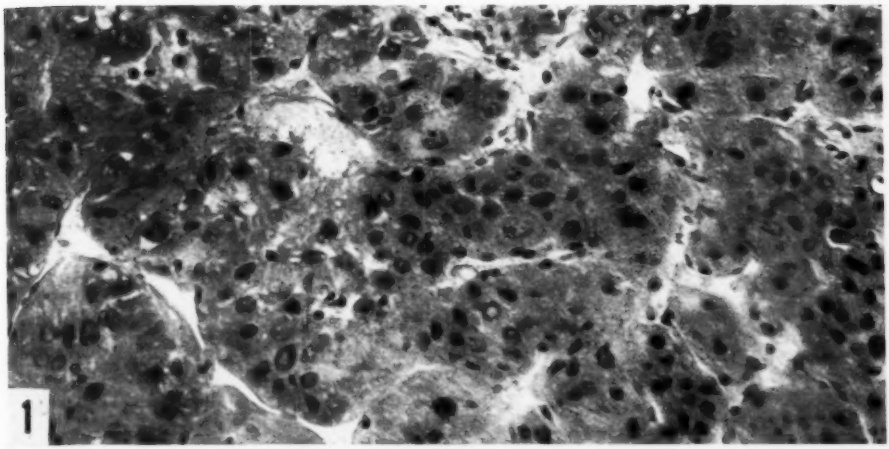
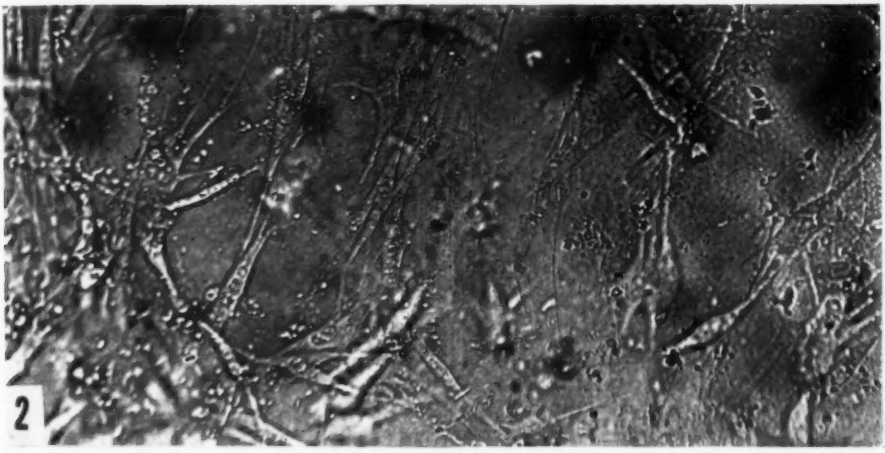
Plate 104

Figure 1.—Section of hepatoma tissue used to plant tissue cultures. Hematoxylin and eosin. × 300

Figure 2.—Area of culture of hepatoma tissue after 176 days and 5 transplantations *in vitro*. This culture was photographed 15 days after the last transplantation. × 200

Figure 3.—Section of sarcoma arising from injection of hepatoma tissue culture. Hematoxylin and eosin. × 300





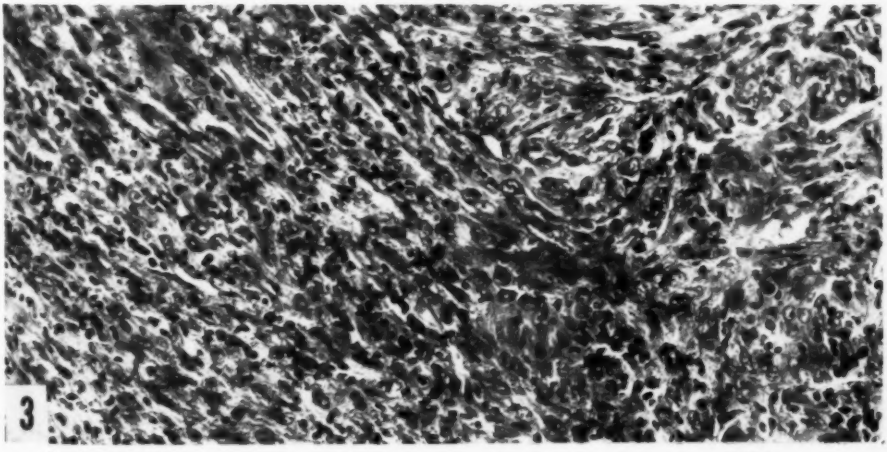


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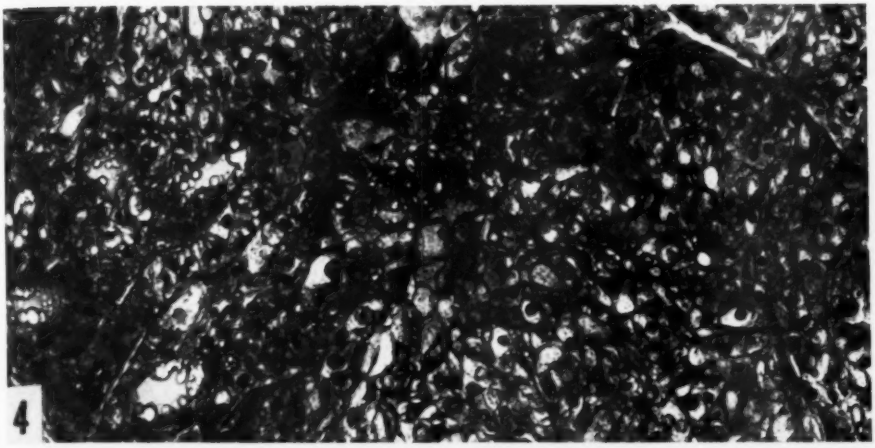
PLATE 105

FIGURE 4.—Section of thyroid tumor used to plant tissue cultures of series 1,251. Hematoxylin and eosin. × 300

FIGURE 5.—Area of culture of thyroid tumor tissue of series 1,251 after 214 days and 9 transplantations *in vitro* and 12 days after last transplantation. × 200

FIGURE 6.—Section of sarcoma arising from injection of the culture of figure 5. Note the segregation of muscle bundles by invading tumor cells. Hematoxylin and eosin. × 300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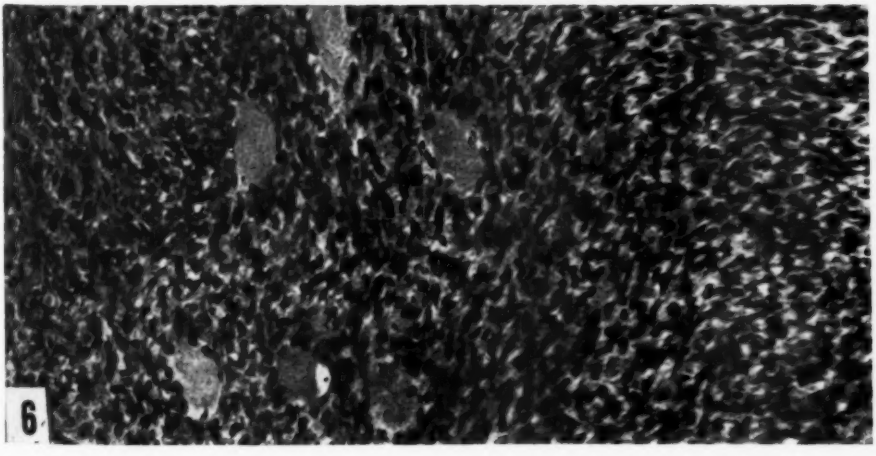
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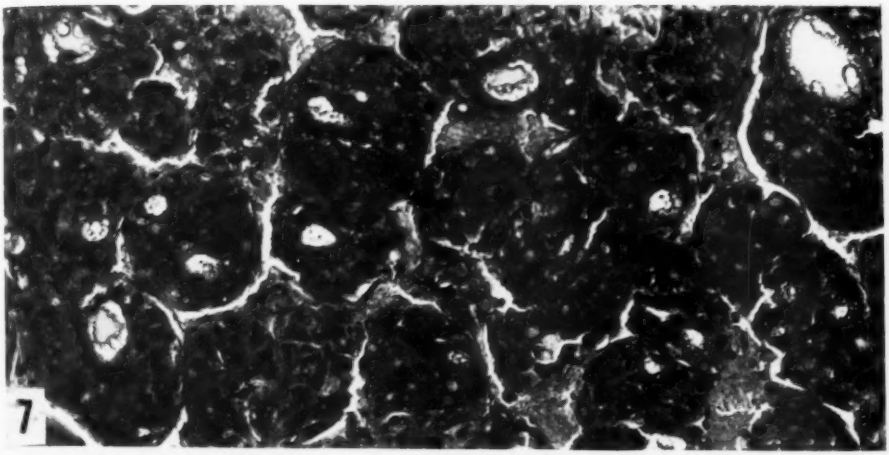
PLATE 106

FIGURE 7.—Section of thyroid tumor used to plant tissue cultures of series 1,252. Hematoxylin and eosin. × 300

FIGURE 8.—Area of culture of thyroid tumor tissue of series 1,252 after 249 days and 8 transplantations *in vitro* and 47 days after last transplantation. × 200

FIGURE 9.—Section of sarcoma arising from injection of the culture of figure 8. Note the segregation of muscle bundles by invading tumor cells. Hematoxylin and eosin. × 300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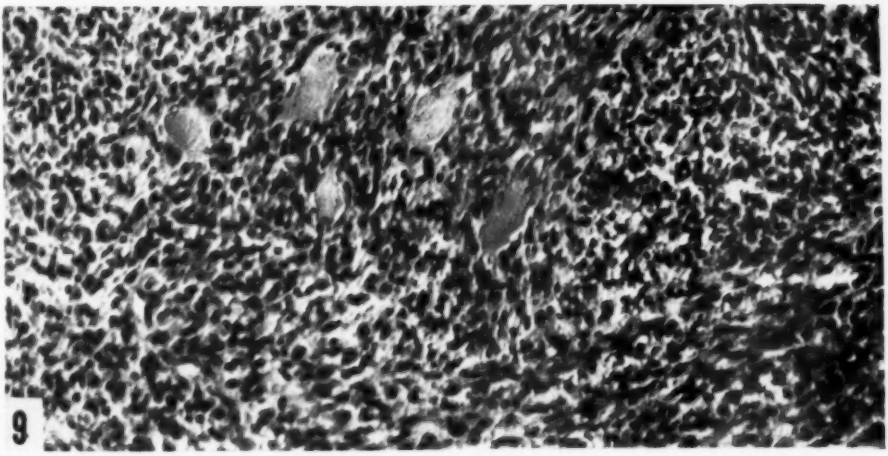
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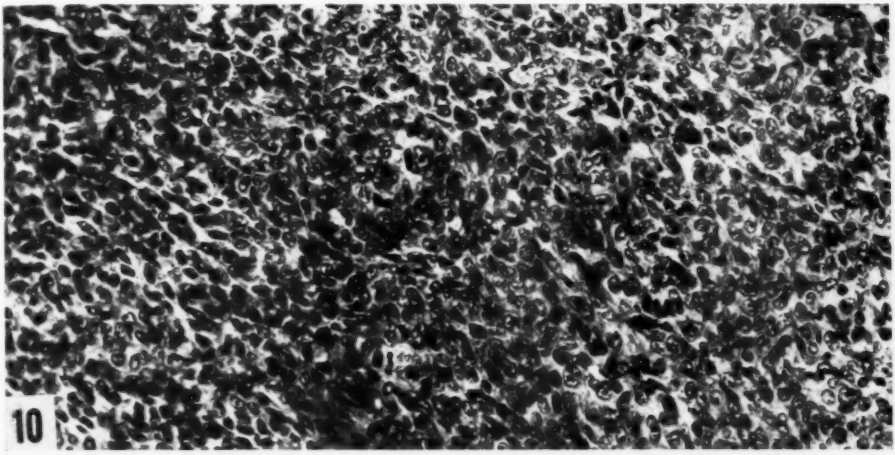
PLATE 107

FIGURE 10.—Section of Cloudman melanoma S91 tissue used to plant tissue cultures of series 400. Hematoxylin and eosin. × 300

FIGURE 11.—Area of culture of melanoma tissue of series 400 after 252 days and 2 transplantations *in vitro* and 26 days after last transplantation. × 200

FIGURE 12.—Section of sarcoma or amelanotic melanoma, arising from injection of melanoma tissue culture. Note the segregation of muscle bundles by invading tumor cells. Hematoxylin and eosin. × 300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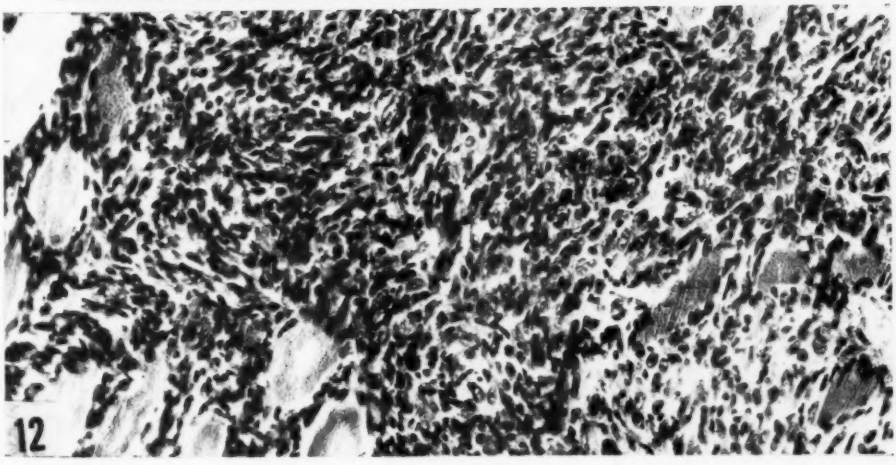
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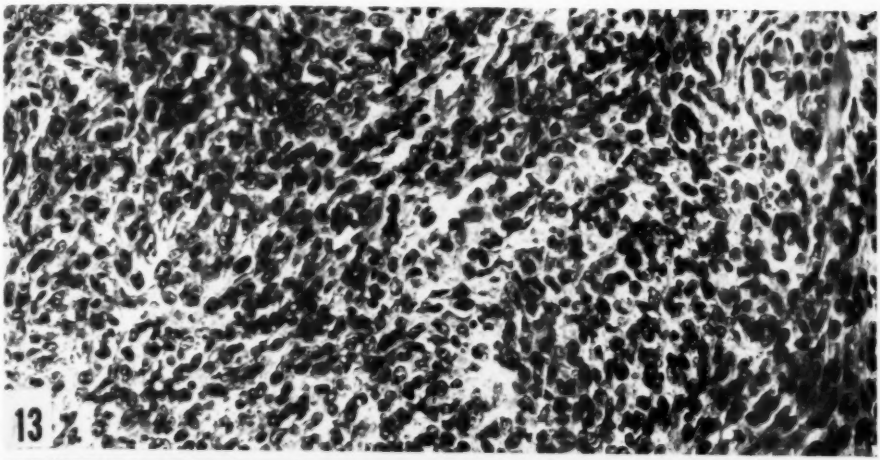
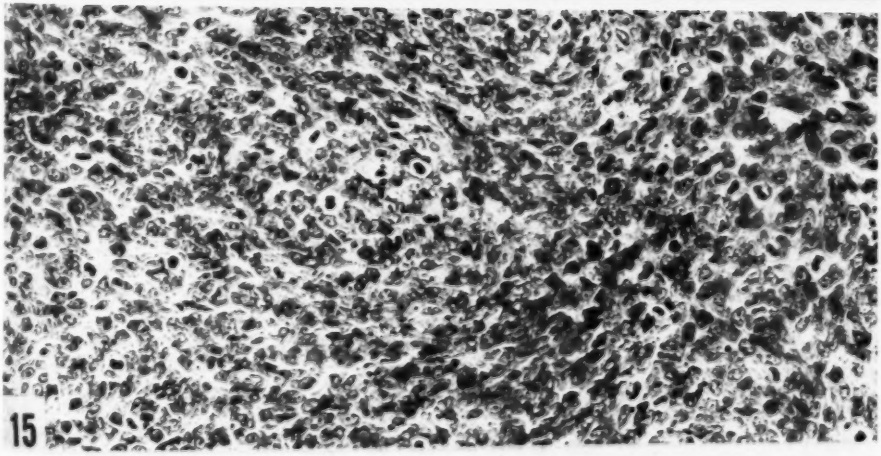
PLATE 108

FIGURE 13.—Section of Cloudman amelanotic melanoma used to plant tissue cultures of series 401. Hematoxylin and eosin. × 300

FIGURE 14.—Area of amelanotic melanoma tissue culture after 252 days and 2 transplantations *in vitro* and 50 days after last transplantation. × 200

FIGURE 15.—Section of melanotic melanoma arising from injection of amelanotic melanoma tissue culture. Hematoxylin and eosin. × 300





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GROWTH AND HISTOPATHOLOGY OF MELANOTIC AND AMELANOTIC DERIVATIVES OF THE CLOUDMAN MELANOMA S91[1]

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The present paper gives a general and detailed account of the growth and histopathology of the melanotic and amelanotic derivatives of the Cloudman melanoma S91.

In previously reported work (*1*) brief mention was made of a partially pigment-free line of the Cloudman melanoma S91, which was obtained after transfer of the pigmented melanoma to strain BALB/c (strain C) mice and deliberate selection of the least pigmented portions of the tumor for subinoculation into other strain C mice. Since these earlier studies, the partially melanotic line has been obtained in a predominantly amelanotic form, and studies have been carried out on the relative rôle of selection and of the genetic background of the host on the development of the melanin in this tumor.

The data here presented are the results of biologic and pathologic studies carried out in the laboratories of the National Cancer Institute. These observations led to many unsolved problems related to melanomas which will be the basis for further investigation, particularly on the relationship between melanin production and mitotic activity in relation to the age of the tumor.

Previous History of the Tumor

Melanoma S91 is a malignant melanoma originating at the base of the tail in a DBA/Jax female mouse, and was first described in 1937 by Dr. A. M. Cloudman of the Roscoe B. Jackson Memorial Laboratory (*2*). It is still being carried in the laboratory of origin and is now in the 144th transplant generation.[4]

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[4] The tumor is transplanted there once a month and kills the host in about 10–12 weeks. Metastases to the lungs frequently occur. Grossly, the melanoma is light gray with dark pigmented spots, fairly firm. Histologically, "the tumor cells are arranged as prominent perivascular mantles or as diffuse cell masses. The cell components are large, oval, polyhedral, or spindle-shaped. Mitotic figures are numerous. Some of the cells contain dark pigment (melanin). There is a great variation in cell arrangement and pigment content in the various transfer generations. The stroma is delicate and vascular." (Personal communication from Dr. E. A. Fekete.)

The tumor was acquired by the National Cancer Institute in November 1939, in the 12th transplant generation of mice of the DBA strain of origin.

METHODS

Tumor transplantation was carried out by trocar inoculation of tissue fragments approximately 2×3×5 mm. All inoculations were made subcutaneously, except as indicated in the text.

For histologic investigation, paraffin sections stained with hematoxylin and eosin were used routinely. In addition, the following special stains and methods were used: van Gieson, Masson's trichrome stain, Heidenhain's azan, Mallory's aniline blue, aldehydefuchsin of Gomori counterstained with Heidenhain's azan in which aniline blue was replaced by light green (*3*), phosphotungstic acid-hematoxylin, periodic acid-Schiff, toluidine blue, Wilder's reticulum stain, paraffin sections bleached with H_2O_2 and counterstained with hematoxylin and eosin, Prussian blue reaction for iron, dopa reaction on frozen sections (*4*, *5*) and on paraffin sections (*6*), nucleoplasmatic stain of del Rio-Hortega (Dr. M. Polak), Krajian silver, and chloral silver nitrate-pyrogallol for neurofibrils adapted for premelanin (*7*). Fresh material was observed under the phase-contrast microscope and cleared with glycerin for observation of melanoblasts in fresh tissue (*8*).

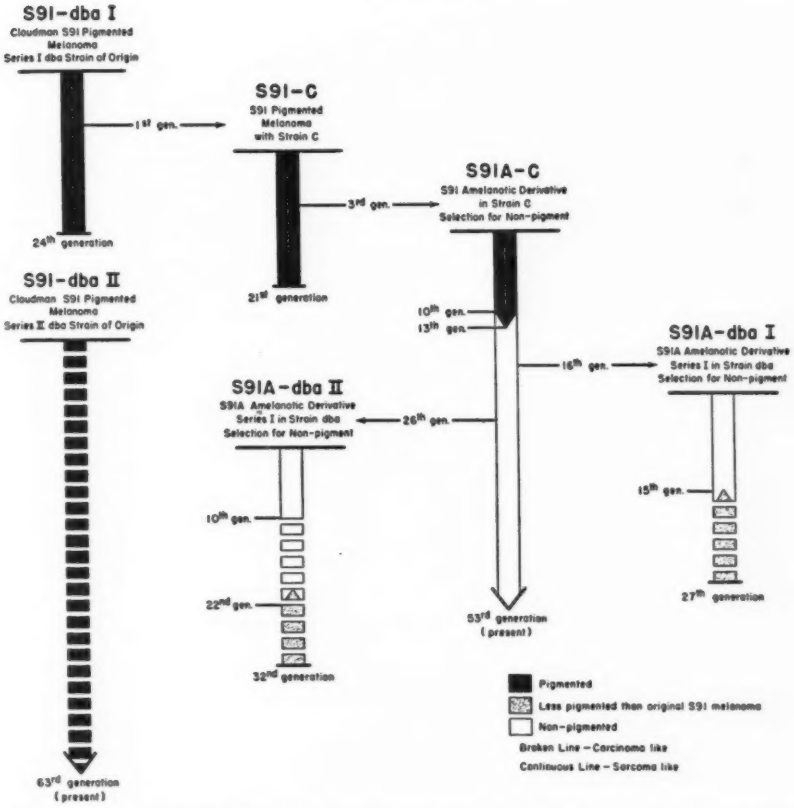
Results of Transplantation Experiments

Text-figure 1 shows the genealogy of the melanotic and amelanotic lines derived from the original Cloudman melanoma S91 and indicates the symbols used in this study. These derivatives are illustrated in figures 1 to 4. Figure 1 shows the S91 melanoma in the DBA strain of origin. Figure 2 is the S91 melanoma in strain C mice. Figure 3 shows the predominantly amelanotic melanoma in strain C obtained by selection for least pigmented areas for transfer, and figure 4 shows the increase in pigmentation of the amelanotic melanoma after return to the pigmented strain of origin (DBA). All transplants were done in males.

The S91–DBA–I was carried subcutaneously for 24 generations in strain DBA and lost by accident in October 1945. All other lines derive from this tumor. The lost tumor was replaced in May 1946 from Cloudman's laboratory. The transplants were carried out subcutaneously until August 1944, the 16th generation, and then intramuscularly. At the present time, the S91–DBA–II melanoma is in the 63d generation.

In October 1941, the S91–DBA–I melanoma from the first generation in this laboratory was transplanted subcutaneoulsy to strain C mice (S91–C). This line was carried for only 21 generations (Jan. 1945). Tumor tissue for transplantation was not selected for pigmented or nonpigmented areas.

In the course of this work, portions of some of the subcutaneous transplants in both strains DBA and C were observed to be less melanotic than the main tumor mass, appearing grayish in color. Transplants of these relatively amelanotic portions were selected from strain C mice in the 3d gen-



TEXT-FIGURE 1.—Derivation of melanotic and amelanotic lines of the Cloudman S91 melanoma.

eration (June 1942) and transplanted into other mice of strain C. This is the origin of the S91A–C melanoma. The transplants were made subcutaneously until (Aug. 1944) the 9th generation and then intramuscularly. Continued selection for nonmelanotic tissue for transplant during subsequent transfer generations resulted in the development of a grossly amelanotic tumor line. The tumor was gray-white until the 13th generation and then became almost entirely white macrosciopcally. A few pigmented cells, however, could frequently be found in microscopic sections of this tumor, and were visible microscopically *in vivo* when grown in the transparent chamber in mice (*9*). The partially amelanotic tumor was obtained in a more amelanotic form when, in the 24th generation, a very small fragment, of approximately 1 sq. mm., was introduced into a transparent chamber in a strain C mouse. This implant grew for 30 days, and no pigment cells were detected. This tissue was then used to carry on the amelanotic lines S91A–C. Figure 5 shows the above-mentioned S91A–C implant growing in the transparent chamber of the mouse, in contrast to the S91–

DBA–II in the 62d generation (fig. 6). It may be noted here that preliminary systematic studies now under investigation have shown that the degree of pigmentation of the S91A–C depends greatly upon the age of the tumor.

After 16 generations, the amelanotic melanoma S91A–C was returned to the strain of origin (DBA) by intramuscular transplantation and designated S91A–DBA–I. In spite of continued careful selection of the grossly least pigmented areas for transfer, growth in the pigmented strain DBA resulted in a sudden increase in pigmentation at about the 14th generation, increasing in grayness from that time. The S91A–DBA–I, however, never became as pigmented as the original tumor. S91A–DBA–I was carried for only 27 generations (Oct. 1945). During this period the S91A–C, which was being carried in nonpigmented strain C mice, maintained a predominantly nonpigmented character.

A repetition of this experiment was carried out by intramuscular transfer of tissue from the 26th generation of S91A–C back to strain DBA mice (June 1948). This tumor line was designated S91A–DBA–II. The amelanotic tumor became grayish in the 9th generation and was noticeably darker after the 22d generation. One animal of each of the 9th, 13th, 14th, 16th, 17th, 19th, and 20th generations was of more advanced age and showed more pigment histologically.

Histology and Biology of the Six Tumor-Transplant Series

General.—First to be considered are the histologic and cytologic properties found present in the six series of tumor transplants. These are considered especially in relation to the reports of other authors. Following this, the particular characteristics of each transplant series are described.

The histology of these tumors corresponds in most respects to the description of human malignant melanomas (*10–13*). Because of the great similarity to be found between human tumors and those of the mouse, some of the histologic descriptions previously made by Dawson (*10*) and Miescher (*11*) are repeated. These similar findings are of theoretical and practical interest in a consideration of the biology of melanomas.

The Cloudman melanoma was histologically polymorphous, and was very rich in melanin that could be bleached with H_2O_2, and did not give a positive iron reaction. Different cell forms and different cell arrangements may be found in the tumor. Two main forms of tumor cells were distinguished: first, a rounded or polygonal cell which constitutes the alveolar or endocrine type as described in the German and French literature, particularly a carcinoma-like structure (fig. 7) (*11*); and second, a spindle-shaped cell which reproduces the fascicular types, resembling a sarcoma (fig. 8). The main histologic (*1*) and cytologic (*14*) characteristics of these melanomas in the mouse have been described in previous papers.

The first type of tumor-cell arrangement occupied a large part of the neoplasm; it was often pure but was sometimes mixed with the second type (fig. 9). Generally, the entire section of the tumor appeared solidly cellular, especially in the periphery. Occasionally, a capsule of connec-

tive tissue formed the border between the tumor and the surrounding muscular tissue. A capsule may separate the tumor from the subcutis. The tumor did not infiltrate the epidermis (fig. 10). In most tumors, however, a connective tissue boundary was missing, and there was a destructive infiltration into the surrounding musculature extending into the spaces between single muscle bundles. Heavily pigmented tumor cells or cells in mitosis infiltrated the muscle tissue spaces. Single muscle bundles were walled in and showed evidence of degeneration. Infiltration of tumor cells directly into a muscle fiber was never seen. Occasional areas of calcification were present in the tumor tissue (fig. 11). The location and form of these areas suggested that it is a secondary calcification in degenerating tissue. In such areas, a calcified osteoid substance sometimes occurred. The tumor infiltrated fat tissue also, extending into the interstitial tissue (fig. 12). Perineural lymph spaces may be infiltrated (fig. 13), and in some cases tumor cells appeared to grow into the nerve fiber (fig. 14). Chronic inflammatory cells were found in tumor areas and around blood vessels. A very intensive mitotic activity of the tumor cells, especially of the rounded or polygonal cells, was easily distinguished. Atypical mitoses were common. Study of hundreds of slides revealed that no regular differences could be found between the number of mitoses in the periphery and the medial parts of the tumor. Atypical nuclei were frequent, as were polynucleated or giant cells (fig. 15). Some cells showed a tendency to phagocytize other cells. The question still unanswered at present is whether or not the phagocytic cells are tumor cells or macrophages (figs. 16 and 17). Only one report has been found that melanoma cells phagocytize other tumor cells, and this was in an amelanotic melanoma in man (*15*). A general phagocytic activity by tumor cells has been described by other authors (*12*, *13*), but there were no specified cases of melanomas. Tumor cells in melanomas were described as phagocytizing red blood cells (*12*), and vacuoles that suggest phagocytic activity occur in the cytoplasm.

Central necrosis was frequent in the larger melanomas, and smaller areas of necrosis or hemorrhage were found irregularly throughout the sections.

This melanoma was generally very poor in connective tissue, especially in the solid areas with rounded or polygonal cells. With the more delicate stains, only a few collagenic fibers were seen between single cells or between groups of cells. More collagen was present in the spindle cell areas. Fine bridges from cell to cell may be found. Elastic fibers were very rare and the reticulum stain did not reveal typical arrangement for this type of neoplasm.

A close relation between tumor cells and blood vessels was seen in round- or polygonal-cell areas. Papillary proliferations of neoplastic cells often seemed to be growing in the lumina of vessels (fig. 18). The tumor cells were in intimate contact with the endothelium of fine capillaries (fig. 19). These formations with radial arrangements of tumor cells around blood vessels closely resembled perithelioma-like structures.

In the classification of malignant melanomas, Dawson (*10*) distinguishes as his third group "the melano-endothelioma and melano-perithelioma, in which in the former sheets of large protoplasmatic cells occur arranged often as in some of the glands of internal secretion, or in the latter, where large cells are arranged on a central papillary or vascular core." One tumor of the series S91A–C, 12th generation, showed a remarkable proliferation of fine blood vessels in some large areas. The whole arrangement resembled a hemangioma-like tumor (fig. 20). In general, the tumor seemed to have a particular tendency to grow around and in larger blood vessels. Tumor-cell infiltration and marked enlargement of the vessel wall may develop. Tumor cells may penetrate into the lumen and give origin to a tumor thrombus (fig. 21). Typical perivascular tumor growth was seen in lung metastases (fig. 22).

The melanin content of the tumor was often very irregular from area to area in the same section. This could be related to the observation that "the dopa-positive cells increase in number and complexity before and during an overproduction of melanin" (*16*). Pack (*17*) believes that very actively growing melanoma cells spend so much of their energy in mitotic activity that the formation of pigment does not occur. A difference in the intensity of mitotic activity was seen in tumors of different ages; younger tumors had more mitoses but different areas of the same tumor showed considerable variation. This observation supports Pack's assumption. Fine melanin granules, however, were found in the cytoplasm of tumor cells in mitosis (fig. 23). The majority of the mitotic figures with melanin granules were in prophase. This finding would seem to contradict Pack's view that the processes of mitosis and melanin formation are not compatible. The question remains, however, whether melanin-containing cells in mitosis are still actively producing melanin. No dopa-positive cells in mitosis have been observed.

Very fine granules of melanin appeared in the tumor cells, regularly or irregularly distributed in the cytoplasm. The probable destiny of the melanin in the tumor cells, based on observations of these tumors and on the review by Kopac (*18*) may be summarized as follows: with increasing age of the cell, melanin granules become larger and conglomerate in the cytoplasm. Pyknotic tumor cells, cells undergoing karyolysis or karyorrhexis were found in many cases to possess fine or large, single or conglomerate, melanin granules in the cell body or in its dendritic processes.

A difference between pigment-producing melanoma cells (probably derived from the normal melanoblasts of the skin) and pigment-phagocytizing cells or macrophages was seen. The latter were located mostly in loose groups in or around necrotic areas; the melanin conglomerates were much heavier and irregular and appeared to be somewhat darker brown in color; the nuclei, when not obscured by melanin, did not show the characteristics of tumor-cell nuclei. In the tumor-cell nuclei, the chromatin structure appeared to be more intense, and the nucleoli seemed to be larger than those of macrophages. This has been shown clearly

by Dalton (*13*) by electronmicroscopy studies. The macrophages phagocytize the melanin granules, which appeared to be free in the intercellular spaces after dissolution of the tumor cells.

A criterion for the differentiation between melanin-producing and melanin-phagocytizing cells is the dopa reaction. In serial sections of the same block, those with the dopa reaction showed a strikingly large number of cells with granules, particularly at the periphery. In his first publication, Bloch wrote that "in malignant melanomas, the cells around the growing margin of the tumor were most apt to be dopa-positive, while many of the cells in the center of the tumor were negative" (*20*).

The dopa-positive granules were much finer and smaller, may have a black color different from the already formed, light brown, melanin granules, which can be seen in forms as described above (fig. 24). Under the phase contrast microscope, the melanin granules in the parathyroid gland of normal strain C58 Black mice showed a discoid form (*8*). Melanin granules of tumor cells range from 0.3 to 1.0 micron according to light microscope observations of Woods *et al.* (14). Dalton (*19*), however, on the basis of electron-microscope studies, states that these particles range in size from 0.1 to 0.3 micron. It is possible that all the melanoma cells do not give a positive dopa reaction, in which case a differentiation from macrophages is not always easy. The dopa reaction was described many years ago by Bloch and summarized by Laidlaw (*20*). Large quantities of melanin may occur in macrophages located in necrotic zones, where contiguous living tumor cells contain only a very small quantity of pigment. No intranuclear melanin production was observed in this tumor as has been indicated in some reports (*12*).

Different shapes of the melanoma cells can be distinguished with the dopa reaction similar to those revealed by Cajal's silver impregnation for premelanin, or to those seen by the phase microscope. With these methods it is possible to detect round, spindle-like (fig. 25) and dendritic melanin-producing tumor cells (figs. 26, 27, and 28). This was stated clearly in 1932 by Laidlaw (*20*) who also said "the shape of the cells (melanoma cells) has absolutely nothing to do with its melanin producing power or with its malignancy." Transitions between those forms are frequent and do not always show a typical shape, especially the dendritic cells, as described in the skin of the mouse (*21*) or of man (*11*, *22*).

Individual tumor lines; biologic behavior and histology.—This is a somewhat generalized presentation of the characteristics of the tumor, and it is obvious that considerable variation may occcur from generation to generation, from animal to animal of the same generation, or even in a single slide.

(1) Melanoma S91 in strain DBA mice (series I) from the 1st to the 24th generation

The tumor transplants "took" successfully in approximately 100 percent of the cases. The tumors grew rapidly in comparison with growth rates of the amelanotic lines, killing the host after an average interval of 90 days

(39 animals). Most of the tumors metastasized to the lungs (fig. 29) after about 70 days.

Histologically, the pattern was sarcoma-like, and the cells were heavily pigmented. The number of mitoses was inversely proportional to the degree of pigmentation. Necrosis and hemorrhage were rare, and an inflammatory reaction was marked only where considerable necrosis was present. There were a few perithelioma-like formations.

(2) Melanoma S91 in strain DBA mice (series II) from the 1st to the 53d generation

The tumor transplants "took" in approximately 100 percent of the cases. The growth rate of the tumor was more rapid than in the S91–DBA–I series and it killed the animals after an average interval of 54 days (49 animals). Strain DBA mice, which were less vigorous than those of the preceding series, may account in part for the differences in growth rates and average time of death of these two series.

Histologically, the pattern was carcinoma-like and differed from the S91–DBA–I. The intramuscular transplants invaded the perineural lymph spaces and fat tissue more frequently than the tumors in the preceding series. The pigment quantity and mitotic rate were similar to series I, although the tumor grew faster. Necrosis seemed to be more frequent, particularly after a tumor-age of 1 month, and hemorrhage as well as the number of blood vessels increased with the tumor-growth. This may be related to the more rapid growth rate. Perithelioma-like formations were more frequent than in series I.

(3) Melanoma S91 in strain C mice from the 1st to the 20th generation

After the initial transfer the tumor "took" in 80 to 100 percent of the animals, killing the host after an average interval of 98 days (16 animals). This time corresponds roughly to the lethal effect of the tumor S91–DBA–I. However, there was more variation in the time of death in the strain C mice than in strain DBA. Lung metastases were rarely found earlier than 3 months after transplantation.

Histologically, spindle cells were predominant. Sometimes they were mixed with carcinoma-like structures. The quantity of pigment was inversely proportional to the mitotic rate. The total number of mitoses seemed to be smaller than in the preceding series of DBA mice. The rounded or polygonal cells showed more mitoses than the melanin-containing spindle cells. An increased amount of pigment was present between the 9th and 17th generations. Pigment could be found spread irregularly through a section and large, completely pigment-free areas were seen microscopically. Hemorrhage was less frequent than in DBA mice; the number of vessels and perithelioma-like structures was generally large. Necrosis seemed to increase with the tumor age. The lung metastases were mostly subpleural and were rarely found around bronchi. They were often overloaded with pigment. Occasionally, the

metastases showed typical tumor growth but sometimes single tumor cells, with or without pigment, were found lying without reaction in the alveolar septa or lumina.

(4) Melanoma S91A in strain C mice from the 1st to the 46th generation

The tumor transplants "took" in 50 to 70 percent of the cases, killing the host after an average interval of 123 days (45 animals). The S91A melanoma produced fewer metastases than the S91. Only 9 metastases to the lungs were observed in the 46 generations. Of these, 7 occurred after 120 days.

Histologically, the tumor showed a marked predominance of spindle cells. These cells were generally shorter than those in the preceding series. Sporadically, carcinoma-like patterns were present. Perithelioma-like formations were present; the spindle cells often had a circular rather than radial arrangement around the blood vessels. After 17 to 19 generations, the number of vessels in the tumor was small. Necroses were more frequent than in S91–C and were sometimes very large. No clear correlation with the slow growth rate could be stated. Intramuscular transplants showed a more intensive destructive infiltration of the musculature; subcutaneous transplants infiltrated more frequently into the fat tissue.

The striking difference between this and the preceding series was the behavior of the melanin content of the tumor. Up to the 9th and 10th generations there was still heavy pigmentation. This decreased more or less abruptly, and after the 14th generation only a few pigmented cells were found in some of the many slides of the 15th, 27th, 34th, 35th, and 37th generations. All other generations were pigment-free, up to and including the last. In tumors of the 16th generation, Krajian's silver technic showed a completely negative reaction. With the dopa reaction (6 hours) a few positive cells were demonstrated. In tumors of the 33d generation, the nucleoplasmic stain of del Rio-Hortega, as modified by Dr. M. Polak, did not demonstrate silver-reducing granules in the protoplasm. In numerous sections taken through a tumor in the 44th generation, some isolated dopa-positive cells were found, but these melanoma cells were very sparsely distributed in restricted areas. There were some spindle-cell-like and some dendritic tumor cells. Melanoma cells showed a difference in intensity of dopa reaction (figs. 30 and 31) within the same section; no melanin was present.

The number of mitoses was generally greater than in S91 in DBA and increased as the tumor became more amelanotic after the 14th generation.

Histologically, the metastases in the lungs and medulla of the adrenal gland of this series confirmed the fact, known from human pathology, that the pigmentation in metastases is not parallel with that of the primary tumor. The number of mitoses was also very variable.

(5) Melanoma S91A in strain DBA mice (series I) from the 1st to the 26th generation

After the first generation the tumor "took" in 100 percent of the cases, killing the animals after an average interval of 52 days (31 animals). No metastases appeared but the animals were killed at a young age. The oldest tumor-bearing animal killed was 180 days of age.

Histologically, the tumor showed a sarcoma-like pattern up to the 19th generation and then was more carcinoma-like. Melanin started to reappear at the 15th generation (with the exception of a few pigmented cells in 1 animal of the 6th generation). The pigmentation seemed to be increased more or less regularly up to the 26th generation but the tumor never became as heavily pigmented as the original. The mitotic activity was intense (all young tumors) and was inversely proportional to the melanin content. In the 2d generation only a few melanoma cells gave a weak dopa reaction (4 hours); Krajian's method revealed no silver-reducing granules in the cytoplasm of tumor cells. The tumor was well vascularized throughout the generations, and perithelioma-like patterns were present. Necrosis was rare, hemorrhage seemed to be increased in the later generations, particularly after the 19th. Growth in musculature and perineural lymph spaces was observed.

(6) Melanoma S91A in strain DBA mice (series II) from the 1st to the 30th generation

The transplants took in 80 to 100 percent of the cases, killing the host after an average interval of 52 days (33 animals). No metastases appeared, the animals were killed while young, the oldest being 150 days of age.

Histologically, the tumor was sarcoma-like until the 10th generation and then was predominantly carcinoma-like, except in the 22d and 24th generation. The tumor infiltrated the musculature. One animal of each of the 9th, 13th, 14th, 16th, 17th, 19th, and 20th generations was killed when the tumor had grown for a longer time in the host animal. These mice showed pigmentation. A persistent light pigmentation was reached at the 22d generation. The mitotic activity was high throughout the generations. In the 8th generation no premelanin or melanin could be demonstrated by the silver impregnation technic of del Rio-Hortega, as modified by Dr. M. Polak. In the 25th generation, the number of melanin-containing cells was very large. Melanoma cells could be demonstrated easily with the dopa reaction; Krajian's silver method revealed many dendritic forms. Necrosis and hemorrhage were infrequent; perithelioma-like formations were less frequent than in S91A-DBA-I.

DISCUSSION

Histogenesis and terminology of melanomas are omitted from this discussion. These have been covered in previous papers (*6*, *10*, *11*, *12*, *13*, *22*). Biochemical and enzymatic data have also been published (*18* and *23–35*).

The observations on the behavior of melanin production of the melanoma S91A in mice of strains C and DBA led to the assumption that

there is a particular influence of the host on these tumors. In a series of transplants of S91 in strains C and DBA mice, it was noted grossly that less pigmented areas appeared spontaneously. Probably many factors are responsible for this change. These factors may be related on the one hand to the peculiarities of the tumor itself, and on the other hand to the influence of the host on the tumor. They seem to be in close relationship, in a sort of reciprocal equilibrium. In the series of transplants of S91A in strain C mice, the particular properties of the tumor can be shown by the fact that even though macroscopically white tumor areas were deliberately chosen for transplant, the growing tumor maintained the faculty of producing melanin for a certain length of time. This can be partly related to the observation that even macroscopically completely white pieces of tumor contain some pigmented cells which are demonstrable either in histological slides or on living tumor tissue in the transparent chamber of the mouse. The transplanted pigmented or nonpigmented melanoma cells are proliferating and still producing melanin. This peculiarity is strongly diminished after the 14th generation of transplant of S91A in strain C mice. The lack of pigmentation in this tumor after the 14th generation of transplant could be explained if the transplanted tumor masses were younger than the preceding series. It has been observed in the present transplant series and from preliminary experiments that young melanomas have less melanin. However, the very old amelanotic tumors may show small foci of melanin. The transplants of S91A in strain C mice were all done at approximately the same age, so that the age factor can be considered constant and of no particular direct influence on the melanin production in this series. Therefore, the lack of pigment production after the 14th generation of combined selection of nonpigmented S91A in strain C mice could be dependent on: (*a*) continued active inhibition from the host, (*b*) continued biologic inadequacy of the host, or (*c*) progressive diminution of the tumor autonomy. Of particular interest is the fact that melanoma cells were still found, although weakly dopa-positive, and that they were not able to produce melanin consistently. It was pointed out that even in the same section, under identical conditions, melanoma cells showed a different intensity of the dopa reaction. In addition, premelanin can be found.

These findings appear to be in disagreement with the assumption that "where there is no melanin, there are no dopa-positive cells" (*20*). However, it was reported later (*17*) that a dopa-positive reaction is present even in nonpigmented human melanomas.

Points that still need clarification are the discrepancies in the histologic and biochemic enzymologic findings. Histologically, dopa-positive reactions appear in slides of S91A in strain C mice; biochemically, no evidence for dopa oxidase has been found in the same type of tumor (*30, 31, 34*). This can be explained either by the fact that the histochemical results are dealing with artifacts (formalinization of the tissues; autoxidation of the dopa reagents) or that the enzyme found histologically in only a few cells is quantitatively not sufficient to give a measurable biochemical

result. This apparent incongruence may be explained by combined histologic and biochemic studies through a new series of transplants, using homogenized tumors of the same age of S91A in strain C mice, and of combined investigation in one single generation with tumors of progressive ages.

The series of transplants of S91A in strain C mice demonstrate only that it is possible under particular conditions to obtain from a melanotic tumor an amelanotic tumor in mice. This transformation is reversible. If amelanotic tumor fragments are transplanted back into the original pigmented strain DBA host, the tumor will reacquire the ability to produce melanin more or less regularly, in spite of continued selection for nonpigment, although not so intensively as the original S91 tumor. This return of pigmentation appears in S91A-DBA–I after 15 to 16 generations. In the second series of S91A–DBA the pigmentation does not appear as suddenly and shows its dependence on tumor age. The question may arise as to whether or not there are two types of cells present in the original S91 melanoma, and whether these, when selectively transplanted, give rise to the melanotic and amelanotic lines. No morphologic evidence that would clarify this point was found.

A comparison of the two series S91 and S91A in strain C mice and S91A–I and S91A–II in strain DBA mice gives support to the assumption that the host animal with different coat pigmentation possibly plays an important and determining rôle in the production of melanin in the Cloudman melanoma. Experiments in this laboratory have not produced an amelanotic line by selection for less-pigmented areas within strain DBA mice. Since it is well known that the pigment patterns of the host are determined by heredity and that "it is probable that what is determined is the distribution of the dopa-positive cells" (*16*), these findings show that hereditary differences (possibly of a nature such as described above under (*a*) or (*b*) of the first paragraph of this Discussion) may influence repigmentation of the melanoma transplant series. If so, these hereditary characteristics may be reversible.

Sugiura (*36*) reported that nonpigmented melanomas could not be produced from the Harding-Passey melanoma. Further investigation of this subject led Sugiura to the conclusion that "the coat color of the animal appeared to have no influence upon the susceptibility, the rate of growth, or the depth of pigmentation of the tumor." Furthermore, the age of the host (normal or castrated males or females) "did not significantly affect the growth of the neoplasms" (*37*). Harding and Passey (*38*) made the same observations in the first 2 years of their transplants. However, they also reported that in July 1927, "one strain of the tumor suddenly assumed a very active phase. To the naked eye, the tumor was distinctly paler, varying from a dark gray in one animal to a pale slate-color in another. Ultimately, by deliberate selection from the paler tumor, we obtained a completely colorless tumor." The pigmented tumor maintains a slow rate of growth, while the rate of growth of the nonpigmented tumor is rapid. Sugiura (*36*) stated, "by careful selection from

rapidly growing tumors, the Harding-Passey melanoma has been propagated through many generations (1928–46) in his laboratory, by successive graftings into albino mice of Rockland and Bagg strains. So far, however, a nonpigmented melanoma has not been produced." The experiments in this laboratory have also given negative results in this regard.

These diverse findings show a basic difference in the biologic behavior of the Cloudman and the Harding-Passey melanomas, not only in regard to the pigmentation, but also in its growth rate. It should be pointed out, however, that the Harding-Passey melanoma is not strain specific in its origin or transplantability, having arisen in a stock mouse, while the Cloudman S91 melanoma originated in a DBA mouse, an inbred strain, and does not grow in all strains of mice.

The growth as measured by time of death of the host animal in the six melanoma lines, has been analyzed statistically.[5] The results are shown in table 1.

TABLE 1.—*Duration of life of animals bearing transplants of melanoma*

Strain	Tumors (Duration of life of animals after transplantation) [1]	
	S91	S91A
DBA: Series I	90±3.5 (39)	52±2.5 (31)
DBA: Series II	54±2.5 (49)	52±2.0 (33)
C	98±7.5 (16)	123±6.0 (45)

[1] The number following the ± sign is the standard error of the mean, and the number in parenthesis is the number of animals observed.

The S91A–DBA–I melanoma does not differ significantly in its lethal effect from the S91 in strain C. The S91–DBA–II seems to have a much more rapid lethal effect. This, as previously mentioned, could be due to the fact that the strain DBA mice used in this series was not very vigorous. The growth rate of the S91A in strain C mice is markedly lower than that of the original tumor, and is also lower than that of back transplanted tumors, S91–DBA–I and II. If diminished vigor is a factor in these strain DBA mice, its precise rôle is difficult to state. This factor seems to be more applicable to the second series, S91A–DBA–II. The same lethal effect in both series suggests that only changes pertinent to the tumor and independent of the strain may be dealt with in this case. The standard error of both tumors in strain C mice is greater than that of the tumor in strain DBA.

The data presented could suggest that the albino host exerts an inhibiting influence not only upon pigment production, but also upon rate of growth of the tumor as measured by time of death of the host animal after tumor implant. The reciprocal interrelation between the influence of the host on the tumor and the reaction of the tumor on the host is difficult to interpret clearly because of the close association of the four previously mentioned factors: (*a*) continued active inhibition from the

[5] For this study we are indebted to Mr. M. A. Schneiderman of the Biometrics Unit of this Institute.

host, (*b*) continued biologic inadequacy of the host to supply the optimum conditions for growth and pigmentation of tumor, (*c*) progressive diminution of the tumor autonomy or the greater dependence of the tumor on conditions supplied by the host, and (*d*) hereditary modifications of the tumor characteristics.

In addition to producing an amelanotic tumor in strain C mice, these tumor transplant experiments seem to have modified the biologic properties of the Cloudman S91 melanoma. The back transplants into strain DBA mice from a tumor carried for many generations in strain C mice show a less pigmented and faster growing tumor than the original. The relationship between mitotic activity and melanin production in the different series is in accord with these tumor modifications. Growth rate as measured by time of death of the host animal after tumor-implantation is not in accord in all cases. These two similar factors are not comparable, however, since no histologic studies were done on tumors of animals that died spontaneously.

SUMMARY

Histopathologic and cytologic studies were made on tissues obtained by transplantation experiments on the Cloudman melanoma S91, which grows in the DBA strain of origin (a pigmented strain) as a predominantly melanotic tumor. This melanoma was successfully transferred and subinoculated in strain C (BALB/c), an albino mouse, where it grew as a pigmented tumor. However, a predominantly amelanotic derivative was obtained in strain C through careful selection of the least pigmented areas for transfer. This tumor has maintained its relatively nonpigmented character for 53 generations of subinoculation into strain C mice, but became more pigmented when returned to strain DBA mice, in which it had originated, in spite of continued selection of least pigmented areas for transfer.

The chief morphologic findings related to melanin production were as follows: Fine melanin granules are distributed throughout the cytoplasm of melanoma cells. Macrophages, however, contain large irregular granules or melanin. The dopa reaction distinguished melanin-producing and melanin-phagocytizing cells in these tumors. The dopa-positive granules appeared more often as black than brown color, characteristic of melanin granules. The amelanotic derivatives in strain C mice were characterized by an almost complete absence of dopa-positive cells. Return of the amelanotic tumor to strain DBA mice produced greater numbers of dopa-positive cells. These results are discussed in relation to genetic and environmental factors in pigment-cell production in mouse melanomas.

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PLATE 109

FIGURE 1.—S91 melanoma in DBA strain of origin. Metastases to lungs. 5th generation; tumor age, 74 days.

FIGURE 2.—S91 melanoma in strain C mouse with metastases to the lungs. 4th generation; tumor age, 138 days.

FIGURE 3.—S91A melanoma in strain C mouse. 27th generation (2d generation from transparent chamber); tumor age, 76 days.

FIGURE 4.—S91A melanoma returned to strain DBA mouse, showing less pigmentation than the original tumor. Compare with figure 1. First series 18th generation; tumor age, 56 days. This is shown in relation to the S91A melanoma in strain C, 24th generation; tumor age, 121 days.

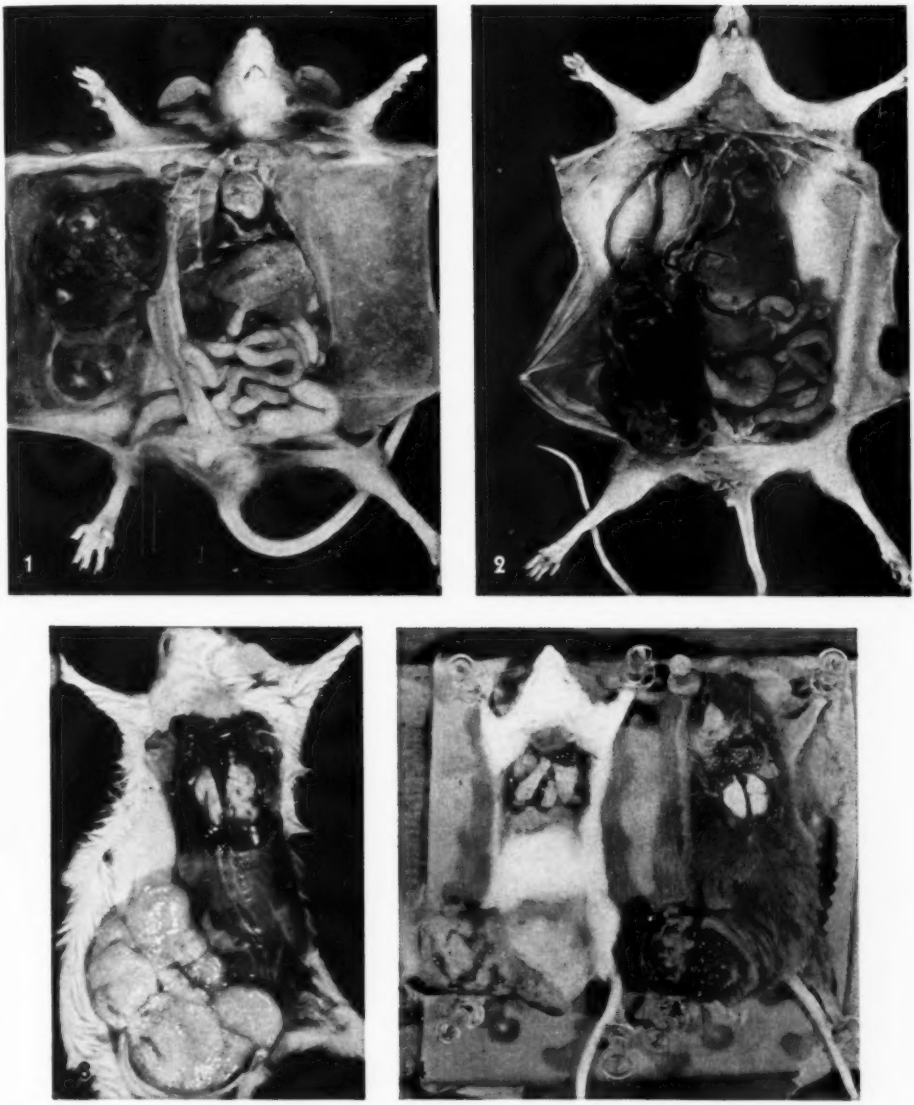

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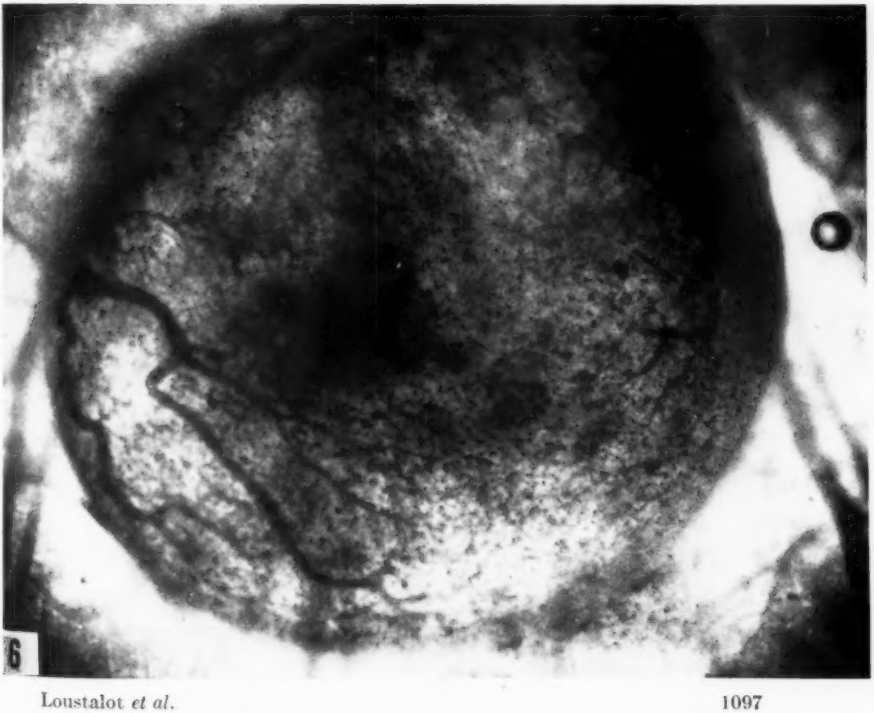
PLATE 110

FIGURE 5.—S91A in strain C mouse in the transparent chamber of the mouse. 25th generation (1st generation in the chamber), tumor age 20 days.

FIGURE 6.—S91–DBA–II in the transparent chamber of the mouse. 62d generation, tumor age 47 days.

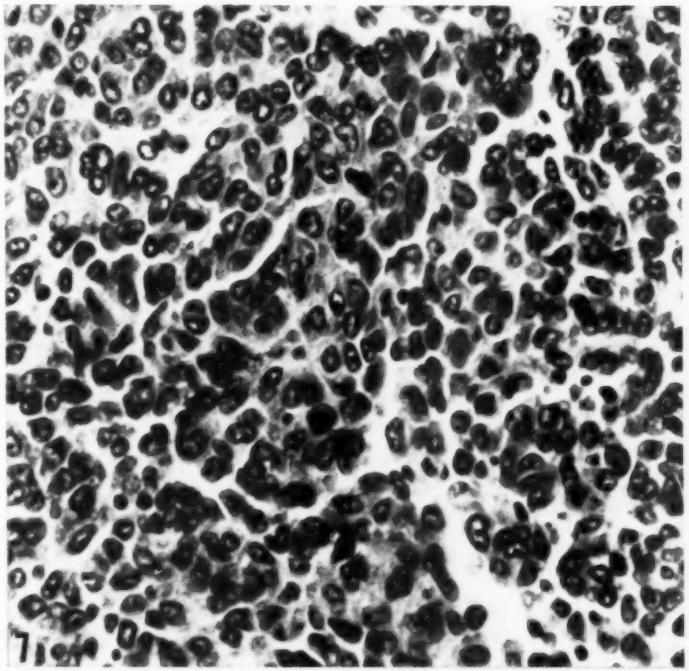


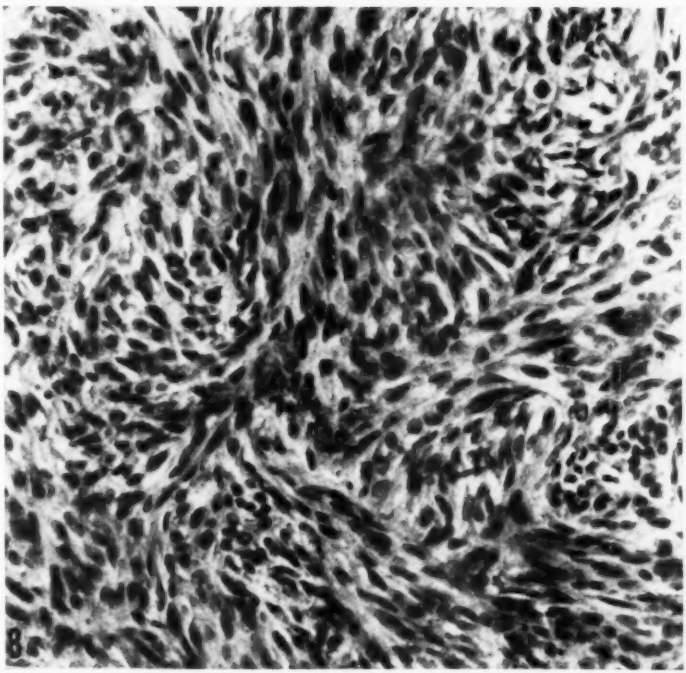
986902—52——20

PLATE 111

FIGURE 7.—Carcinoma-like structure with some alveolar formation. S91-DBA-II, 51st generation. Hematoxylin and eosin. × 310

FIGURE 8.—Sarcoma-like structure; spindle cells; fascicular type. S91-DBA-I, 5th generation. Hematoxylin and eosin. × 300





986902—52——21

PLATE 112

FIGURE 9.—Mixed tumor, with some alveolar and fascicular arrangement. Large number of mitotic figures. S91–C, 5th generation. Hematoxylin and eosin. × 260

FIGURE 10.—Intramuscular transplant showing subcutaneous growth without infiltrating the epidermis. Small necrotic areas at lower right. S91–DBA–II, 15th generation. Hematoxylin and eosin. × 380

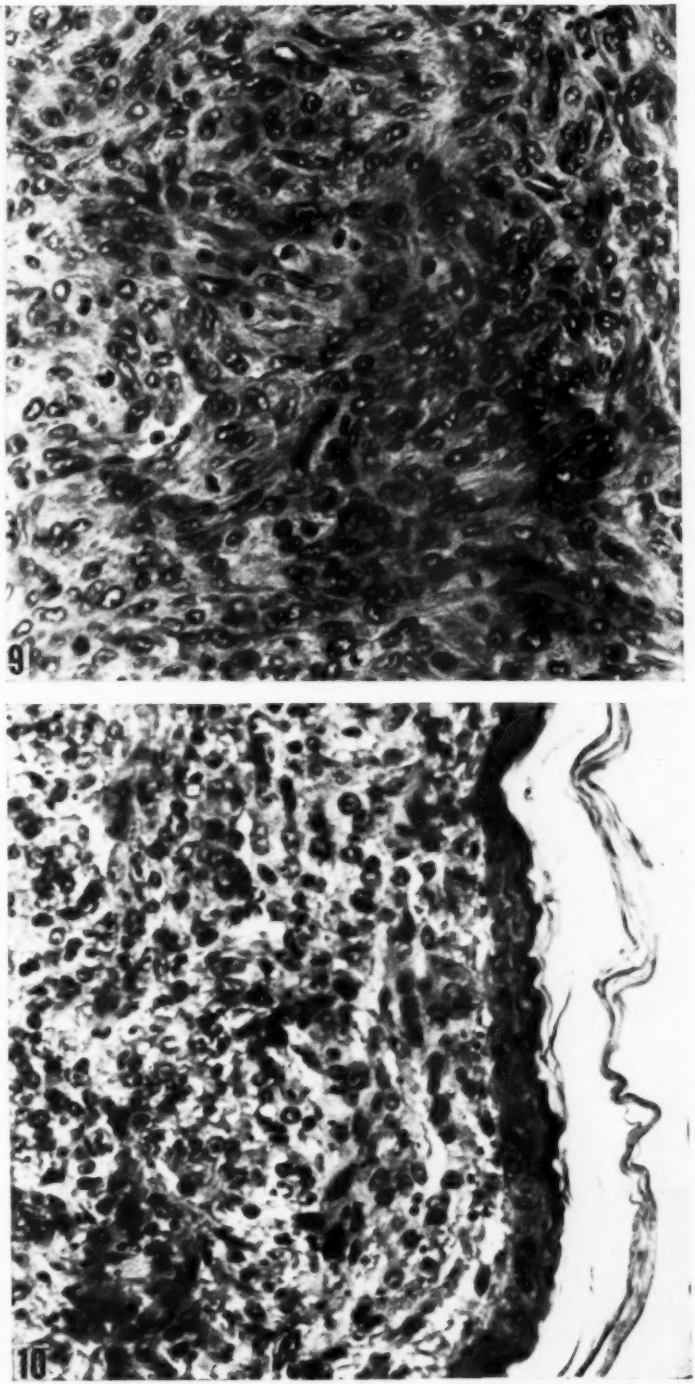

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PLATE 113

FIGURE 11.—Tumor masses infiltrating muscular tissue with two calcified areas. S91–DBA–II, 1st generation. Hematoxylin and eosin. × 320

FIGURE 12.—Infiltration of fat tissue by tumor cells. S91A–C, 39th generation. Hematoxylin and eosin. × 420

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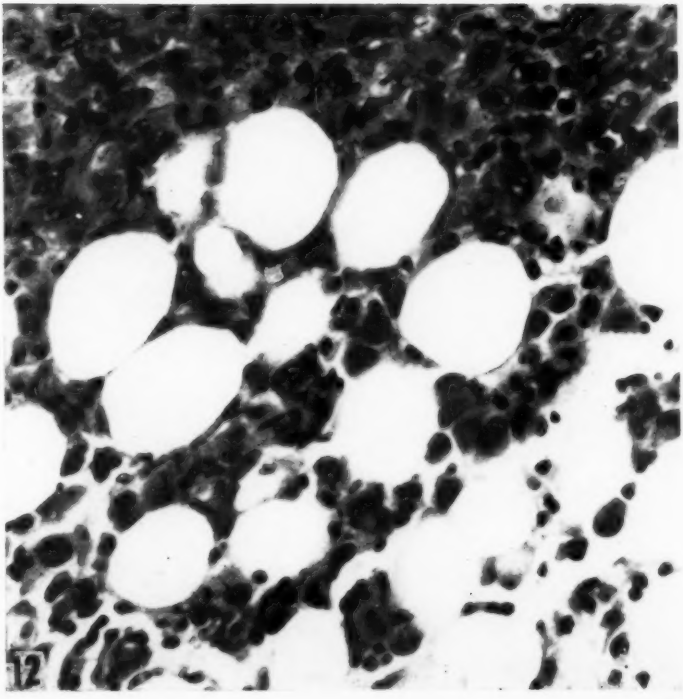
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PLATE 114

FIGURE 13.—Infiltration of tumor cells in perineural lymph space. S91A–DBA–I, 9th generation. Hematoxylin and eosin. × 300

FIGURE 14.—Infiltration of tumor cells into peripheral nerve fibers. S91A–DBA–II, 11th generation. Hematoxylin and eosin. × 360

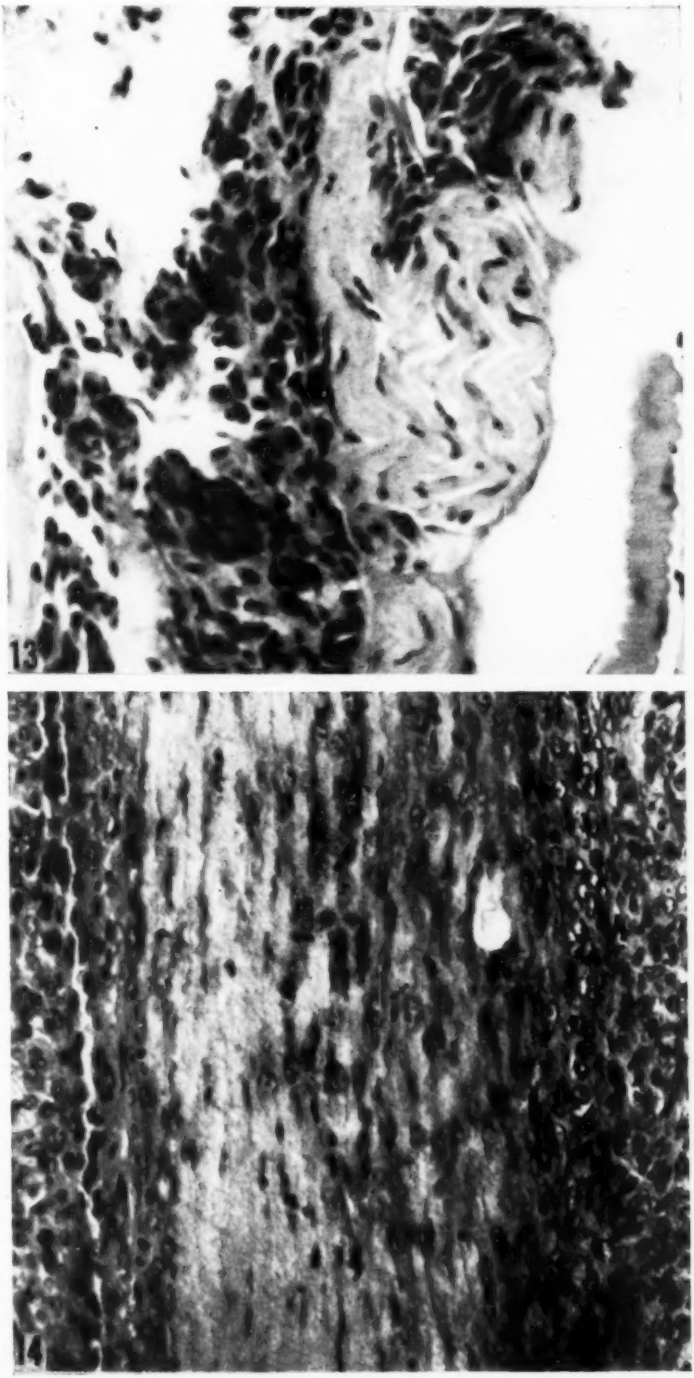


PLATE 115

FIGURE 15.—Three polynucleated cells and one degenerated element, probably phagocytized. S91–DBA–II, 51st generation. Hematoxylin and eosin. × 1,950

Figure 16 may be found in Color Plate 120 at end of plates.

FIGURE 17.—Phagocytosis of one cell with degenerated nucleus. Large melanin granules in the protoplasm and a thin vacuole around the absorbed melanin. S91–DBA–II, 51st generation. Hematoxylin and eosin. × 1,950

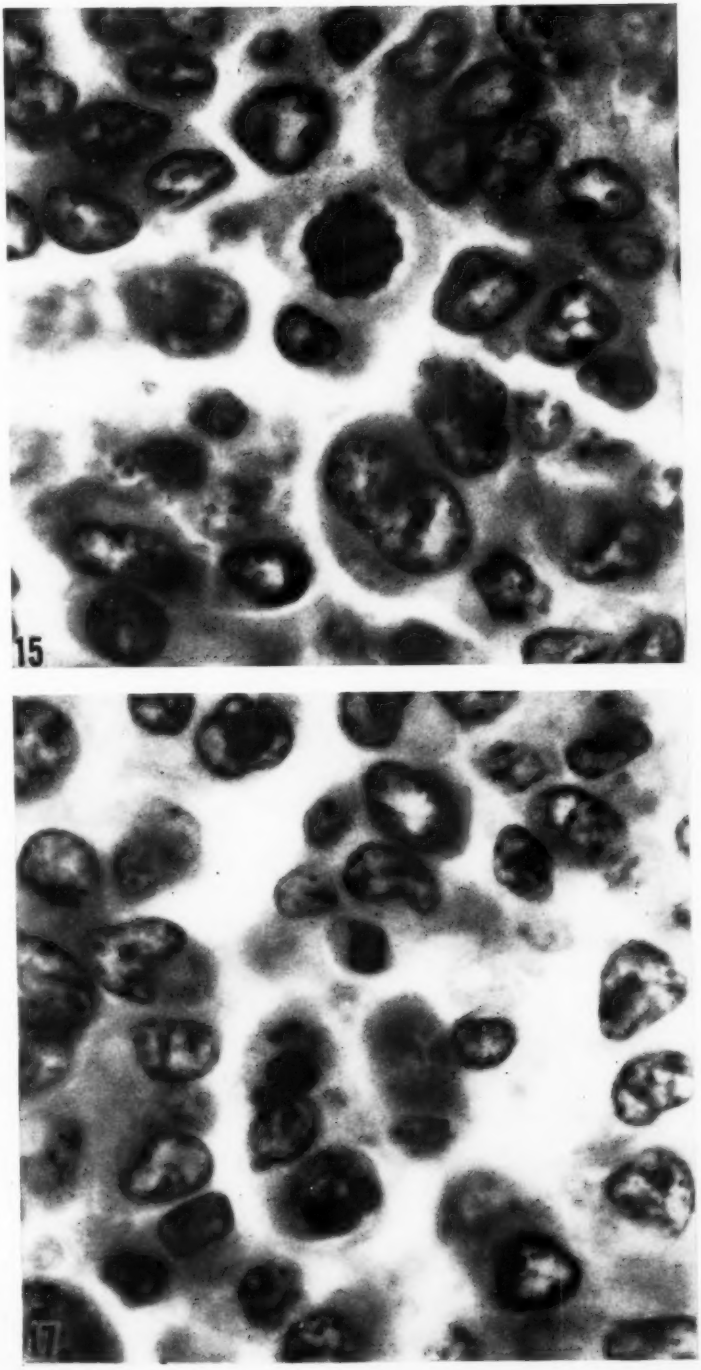


PLATE 116

FIGURE 18.—Papillary proliferation of tumor cells in the lumen of a vessel. S91–DBA–II, 9th generation. Hematoxylin and eosin. × 460

Figure 19 may be found in Color Plate 120 at end of plates.

FIGURE 20.—Hemangioma-like tumor structure with some large capillaries. S91A–C, 12th generation. Hematoxylin and eosin. × 275

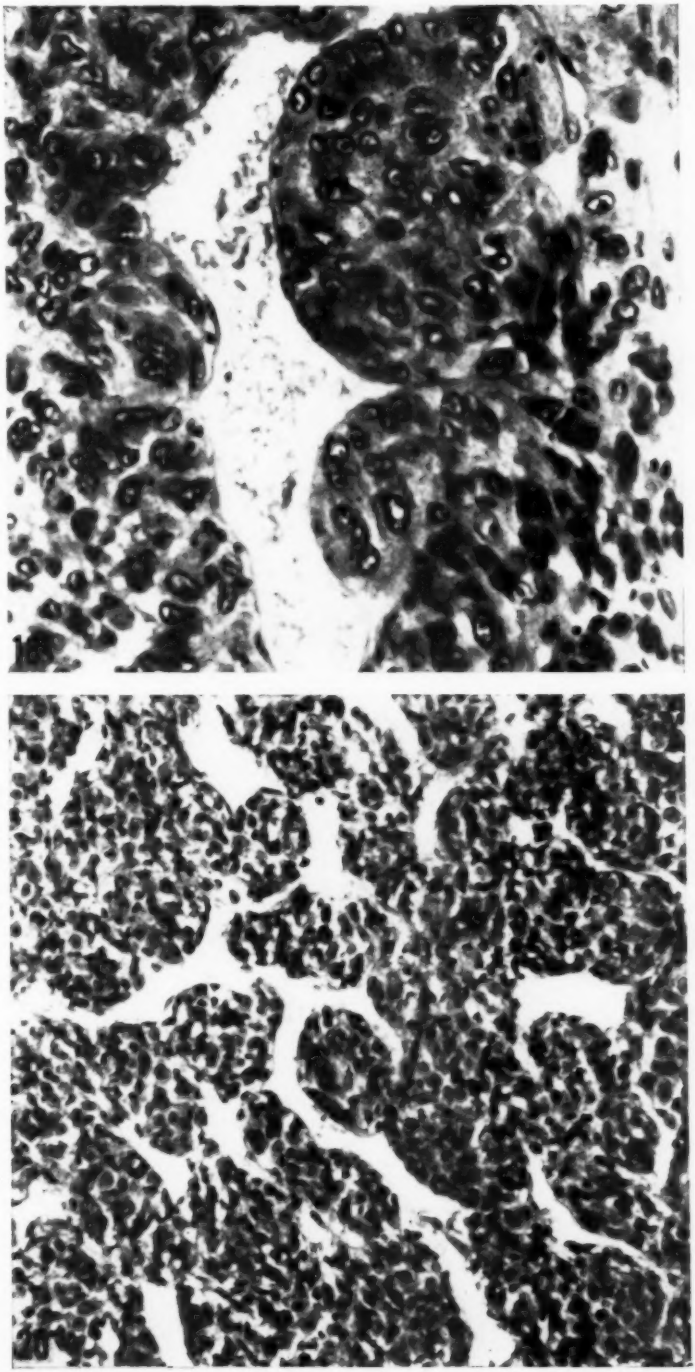


PLATE 117

FIGURE 21.—Infiltration of tumor cells in blood vessel wall and rupture into lumen of vessel, with formation of tumor thrombus. S91A–C, 13th generation. Hematoxylin and eosin. × 165

FIGURE 22.—Perivascular carcinoma-like arrangement of heavily pigmented tumor cells in a lung metastasis. S91–DBA–I, 5th generation. Hematoxylin and eosin. × 290

Figures 23 and 24 may be found in Color Plate 120 at end of plates.

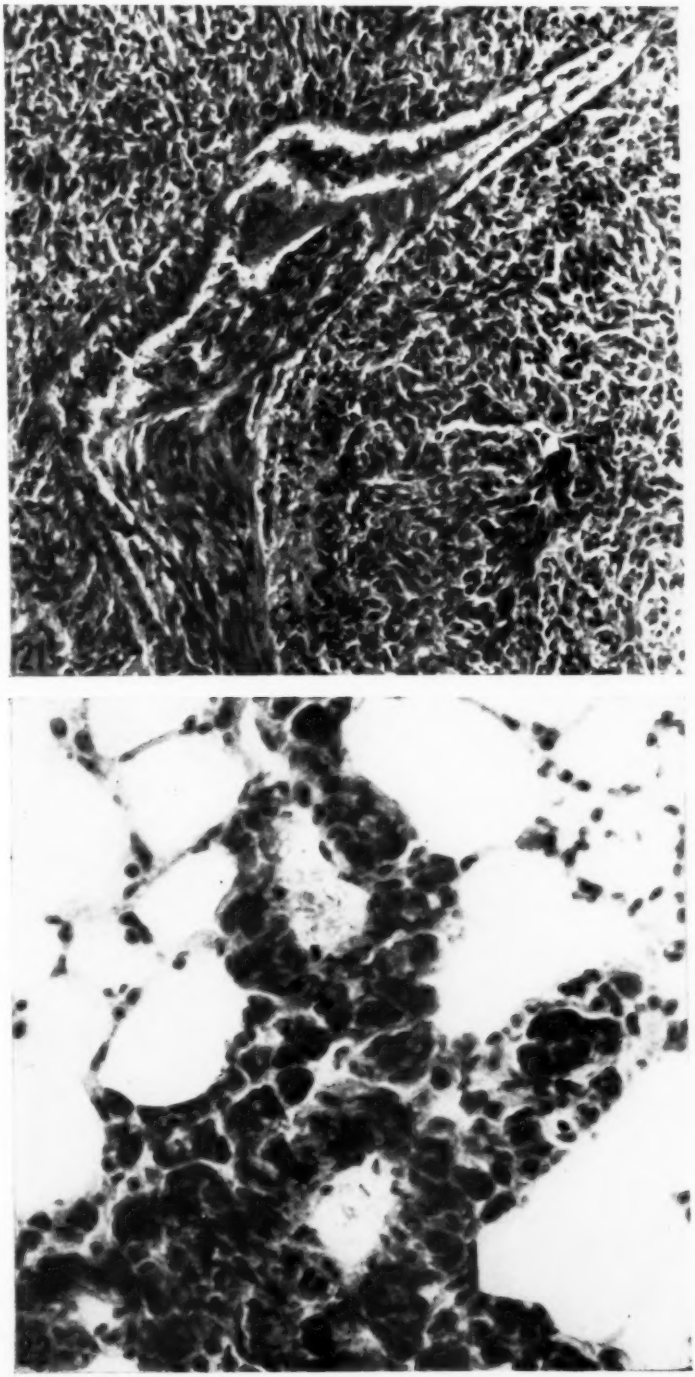
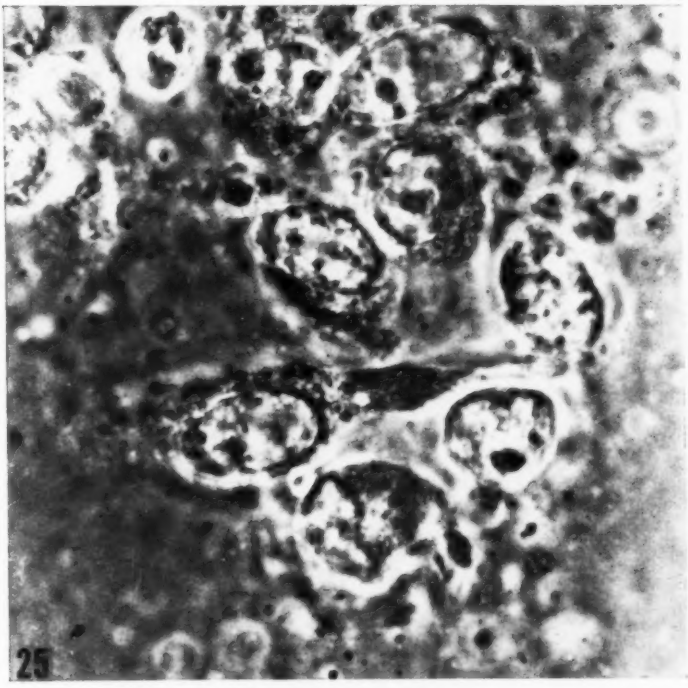


PLATE 118

FIGURE 25.—Spindle-like and round melanoma cells with fine melanin granules. S91–DBA–II, 53d generation. Phase contrast.

FIGURE 26.—Dendritic melanoma cells, dopa reaction. S91A–DBA–II, 16th generation. × 1,340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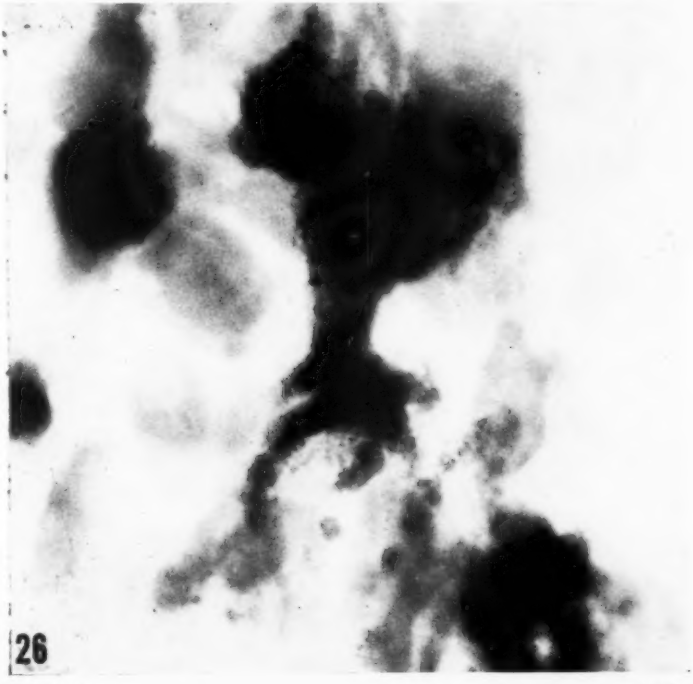
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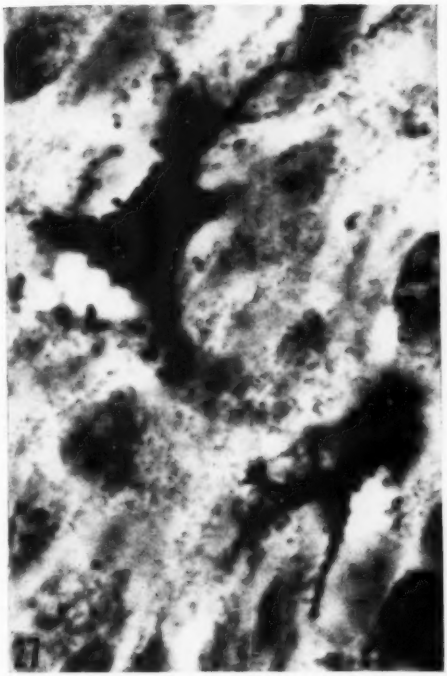
PLATE 119

FIGURE 27.—Two dendritic melanoma cells with premelanin granules. S91A–C, 44th generation. Cajal's silver. × 1,750

FIGURE 28.—Dendritic melanoma cells. S91–DBA–II, 7th generation. Krajian's silver. × 1,500

FIGURE 29.—Multiple subpleural perivascular and peribronchial, heavily pigmented metastases in the lung. S91–DBA–I, 6th generation. Hematoxylin and eosin. × 60



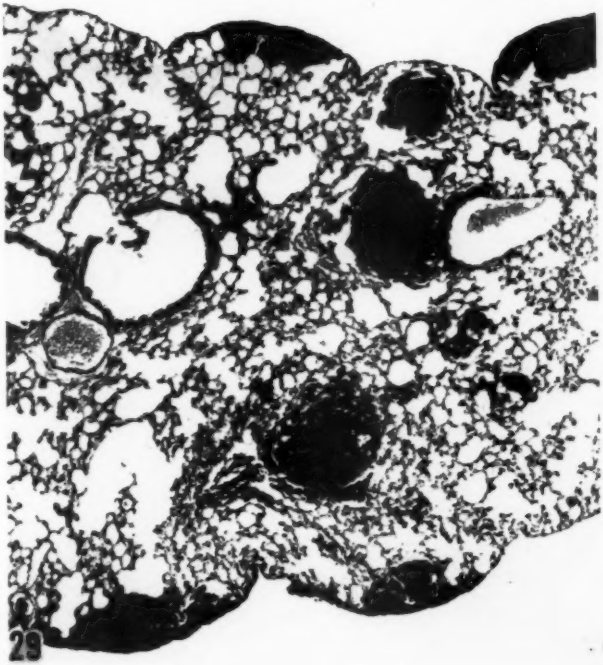


PLATE 120

FIGURE 16.—Phagocytosis of one tumor cell by a macrophage, with large melanin conglomerate in cytoplasm. Note vacuole around phagocytized cell. S91–DBA–II, 51st generation. Hematoxylin and eosin. × 1,950

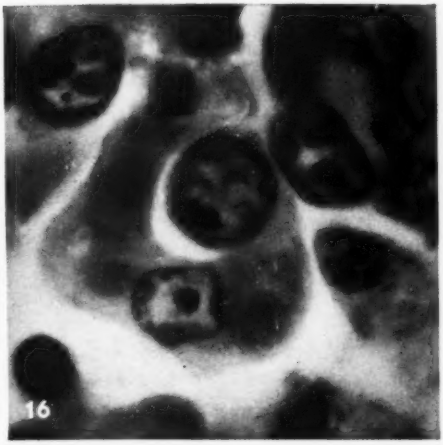
FIGURE 19.—Perithelioma-like formation with some mitotic figures. S91–DBA–II, 51st generation. Hematoxylin and eosin. × 460

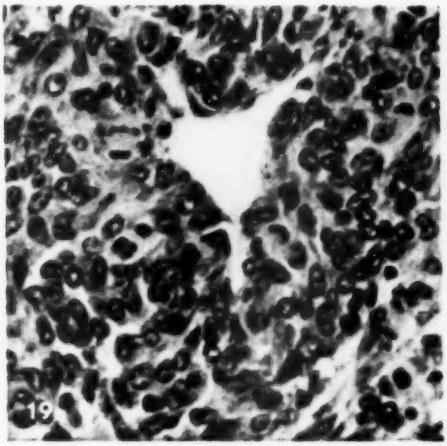
FIGURE 23.—Mitosis in a tumor cell with many fine melanin granules. S91–C, 4th generation. Hematoxylin and eosin. × 1,750

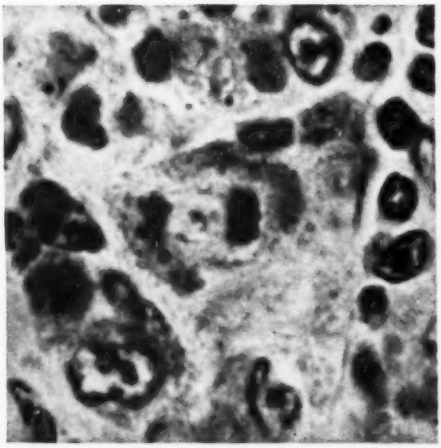
FIGURE 24.—Melanoma cells with black dopa positive granules in contrast to macrophages with large brown melanin conglomerates. S91–DBA–II, 51st generation. Frozen section. × 1,050

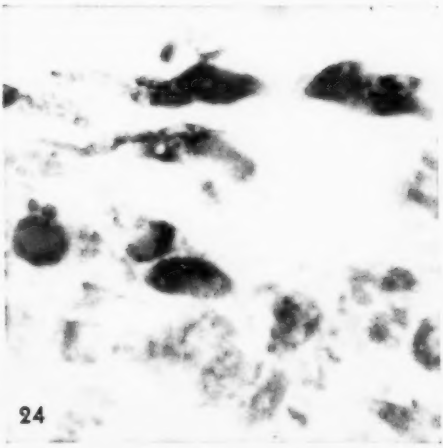
FIGURE 30.—Positive dopa reaction in spindle melanoma cell after 2 hours. S91A–C, 44th generation. × 1,750

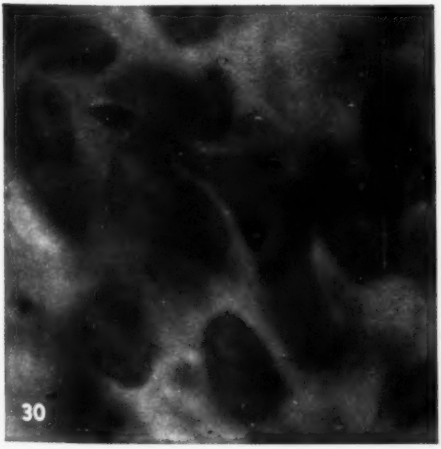
FIGURE 31.—Definite but weaker dopa-positive reaction of a melanoma cell of the same section as figure 30. × 1,750

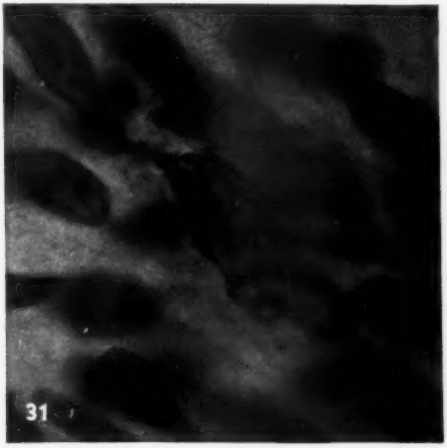












Loustalot *et al.*

THE FLEXED-TAIL-ANEMIA GENE (*f*) AND INDUCED LEUKEMIA IN MICE [1]

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It has been established that genes play a distinct rôle in the etiology of leukemia in mice. Several investigators, notably Slye (*1*), Mercier (*2, 3*) and Hogreffe (*4*), believe that susceptibility to spontaneous leukemia in the mouse is inherited either as a simple recessive or as a simple dominant trait. Serious objections have been raised to the methods used by these workers in arriving at their conclusions (*5, 6*). MacDowell *et al.* (*6, 7, 8*) and Cole and Furth (*9*) have presented evidence to show that the incidence of leukemia in crosses with a low-leukemic strain is roughly correlated with the proportion of total heredity contributed by the high leukemic strain. The most carefully controlled and complete study of genetic influences in spontaneous leukemia (*7*) concerned the crucial test of genetic diversity in a backcross generation in which segregation of genes influencing the incidence of leukemia, would be expected to occur. Genetic diversity of the various progeny in this generation was found, indicating that genes definitely influence the development of leukemia. Additional evidence that genes may modify the expression of leukemia is to be found in linkage tests of known, marked genes and susceptibility to leukemia. Although specific associations of genes and mammary tumors (*10, 11, 12*) and of lung adenomas (*13, 14, 15*) have been reported, only one instance of association of leukemia and a specific gene, dilution (*d*), is known (*6*). Evidence will be presented here to show 1) the existence of a maternal influence responsible for a difference in the incidence of leukemia in reciprocal F_1 hybrid mice, and 2) an association between susceptibility to leukemia and the chromosome carrying the flexed-tail gene (*f*) and a lack of association between leukemia and two other known genes, waved-2 (*wa-2*) and shaker-2 (*sh-2*).

EXPERIMENTAL PROCEDURES

The parent strains of mice used to produce F_1 hybrid and backcross mice segregating for marked genes and leukemia were strain DBA, subline 212, and strain WA.[3] Mice of the F_{28} and F_{29} generations of strain DBA,

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[2] National Institutes of Health, Public Health Service, Federal Security Agency.

[3] The strains WA and DBA were obtained originally from Dr. W. E. Heston. "F_{28} and F_{29} inbred generations of DBA" refers to the degree of inbreeding, since these mice were obtained from Dr. C. C. Little. The WA strain was obtained originally by Dr. W. E. Heston from Dr. G. D. Snell. These mice were inbred only to the F_4 and F_5 when used in the present study.

subline 212 and of the F_4 and F_5 generations of strain WA were used as the nucleus to obtain all animals used in this study. Strain DBA mice are highly susceptible to the development of leukemia following painting with the carcinogen, methylcholanthrene (*16*). In the present study, an incidence of 69.7 percent of leukemia was found in 66 mice painted with a 0.2 percent solution of methylcholanthrene [4] in ethyl ether. Paintings were begun in mice 5 weeks of age and continued, 3 three times a week, for 20 paintings. The mean age at death from leukemia in this group was 9.5 months. Strain WA mice have not been observed to develop leukemia spontaneously in this laboratory. Following painting with methylcholanthrene, 1 in 14 mice developed a typical lymphocytic leukemia at 8 months of age.

Strain WA mice were crossed reciprocally with strain DBA to obtain F_1 hybrid mice. Strain WA is homozygous for three recessive genes, waved-2 (*wa-2*), shaker-2 (*sh-2*) and flexed-tail (*f*). The first two genes are borne on the same chromosome, that is, they are linked (*17*). Flexed-tail is inherited independently of waved-2 and shaker-2. Newborn flexed-tail mice exhibit a transitory, siderocytic anemia (*18, 19*) which disappears for the most part after 2 to 3 weeks. In outcrosses involving this gene, there occurs a deficiency of flexed-tail mice, probably due to the presence of normal overlaps (*20*). Strain DBA carries the normal alleles of these three recessive genes. The F_1 hybrids (DWF_1 and WDF_1) produced were thus heterozygous for the genes studied (*Sh-2 Wa-2/sh-2 wa-2 F/f*). F_1 mice were crossed to strain WA to produce the backcross generation ($DWF_1 \times W$) used to test for linkage. Segregation of the genes studied occurs in the backcross generation so that a 1:1 ratio between waved-2 and normal, shaker-2 and normal, and flexed-tail and normal may be expected. The shaker-2 and waved-2 genes remain together except in the cross-over cases because of the linkage of these genes. In the case of the flexed-tail character, a preponderance of normal individuals is observed as a result of occurrence of normal overlaps. One hundred and sixty-two F_1 mice and 269 backcross mice were produced. These animals were given methylcholanthrene percutaneously beginning at 5 weeks of age, as described previously for the parent strains, were placed 7 in a cage, sexes separately, and given a constant supply of Derwood pellets and water. The backcross mice were identified individually at weaning and classified as to genotype with respect to the three genes under investigation.

Autopsies were done on all animals presenting signs of leukemia or other tumors, those moribund and those found dead. Final diagnoses of leukemia and other tumors were determined from material fixed in Tellyesniczky's fluid and stained with hematoxylin and eosin.[5]

[4] The methylcholanthrene, M. P. 179.9°-180.6° C. corrected, was obtained from Dr. Jonathan Hartwell, National Cancer Institute.

[5] Grateful acknowledgment is made to Dr. Thelma B. Dunn, Laboratory of Pathology, National Cancer Institute, for final diagnosis of leukemic tissues.

RESULTS

One hundred and sixty-two F_1 mice heterozygous for the gene pairs studied were produced. These resulted from reciprocal matings using either a high-leukemic or a low-leukemic mother. Pertinent data relating to incidence of leukemia, mean age at death, other tumor-types, *etc.*, are given in table 1 (groups I and II). Although the strain WA mice used in this cross were not sufficiently inbred to insure genetic homogeneity among the F_1 population, it is of interest to note that a definite maternal influence is evident in the F_1 crosses, such as that described by MacDowell *et al.* (*7, 21*) and by Cole and Furth (*9*), the higher incidence of leukemia appearing when the mother was from the high-leukemic strain, DBA. The difference in incidence of leukemia in the reciprocal F_1 crosses, 18.8 $\pm$7.4 percent, gives a P value of 0.011. It is also of interest to note the greater occurrence of breast adenocarcinomas (42.3 percent) in F_1 mice having the strain DBA mother, while only a small number (7.1 percent) of hybrids of the reciprocal cross of strain WA mother origin possessed such tumors.

In contrast to the relatively early mean age at death from leukemia in strain DBA mice (9.5 months) the mean age at death of the 50 leukemic F_1 mice was 14.9 months. The predominant cell-type of leukemia found in F_1 mice was lymphocytic, being characteristic of strain DBA. In the leukemic F_1 mice, other cell types, such as reticulum-cell sarcoma and "Hodgkin's-like lesions" were also found that were not identified in the high-leukemic parental strain (table 2).

Twenty-seven F_1 females of the ♀ DBA × ♂ WA (DWF_1) cross were used to obtain the DWF_1 × W backcross mice. The 228 mice obtained in this cross represented first and second litters. Forty-one mice were obtained in the reciprocal backcross (♀ WA × ♂ DWF_1 backcross). These mice are not considered, however, in linkage data. Data concerning incidence of leukemia, mean age at death from leukemia, other tumor-types, *etc.*, are listed in table 1 (group III). These data were obtained from mice representing reciprocal backcrosses. Backcross animals, like F_1 hybrids, showed a considerably greater variation at age of death from leukemia and in morphologic variation in cell type. The majority of leukemias in backcross mice were, however, lymphocytic in type (58 of 79). The earliest lymphocytic leukemia appeared at 5 months and the latest at 27 months. There were found, in addition, 4 granulocytic leukemias, 8 reticulum-cell sarcomas, and 9 "Hodgkin's-like lesions." These appeared somewhat later than the typical lymphocytic leukemias (table 2).

Table 3 gives the incidence of leukemia among DWF_1 × W backcross mice classified as to genotype. It is quite clear that there is no relationship between susceptibility to leukemia and either of the linked genes, waved-2 (*w-2*) and shaker-2 (*sh-2*). The flexed-tail (*ff*) segregants, however, were found to be more susceptible to the development of leukemia than their normal (*Ff*) litter mates. Only 28, or 22.8 percent, of normal mice developed leukemia, while 41, or 39 percent, of the flexed-tail mice

TABLE 1.—*Leukemia and other tumors in reciprocal F_1 hybrid and backcross generations of strains DBA and WA*

Group	Cross	Number of mice	Leukemia		Mean age at death; leukemia (months)	Number non-leukemic	Mean age at death; non-leukemic (months)	Other tumors	
			Number	Percent				Type	Number
I	DWF_1	[1] 96	37	38.5	15.2 (8–26)	59	14.9	Breast adenocarcinoma	[2] 22
								Tumor of cervix	1
								Carcinoma of skin	6
								Lung adenoma	20
II	WDF_1	66	13	19.7	17.5 (9–23)	53	19.0	Granulosa cell—ovary	2
								Tumor of cervix	1
								Hepatoma	5
								Carcinoma of skin	4
								Lung adenoma	19
III	$DWF_1 \times W$; $W \times DWF_1$ (combined).	269	79	29.7	14.0 (5–27)	190	14.9	Breast adenocarcinoma	[3] 3
								Lymph-node hemangio-endothelioma	2
								"Mesenteric disease" [4]	2
								Hepatoma	1
								Carcinoma of skin	22
								Lung adenoma	23
								Breast adenocarcinoma	20

[1] Sexes are combined since no sex differences were noted in incidence of leukemia or mean age at death from leukemia.
[2] Adenocarcinoma of the breast appeared in 22 of 52 (42.3 percent) female mice of this cross at a mean age of 12.9 months.
[3] Only 3 adenocarcinomas of the breast appeared in 42 female mice (7.1 percent) at a mean age of 16.6 months.
[4] "Mesenteric disease"—A lesion described by Simonds (*4*) characterized by great enlargement of the mesenteric lymph node, with large blood-filled spaces and atrophy of the usual lymphatic tissue.

TABLE 2.—*Distribution by cell type of leukemia in strain DBA, reciprocal F_1 hybrids and DWF_1–W backcross mice*

Cross	Type of leukemia	Death from leukemia by month																									Total Number	Mean age at death
		3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	
DBA	Lymphocytic	1	1	2	2	5	6	7	6	5	5	3	--	2	1	--	--	--	--	--	--	--	--	--	--	--	--	46	9. 5
WDF_1; DWF_1	Other types of leukemia not found																											
	Lymphocytic	--	--	--	--	--	2	2	2	4	2	2	5	1	1	2	5	3	1	2	--	--	1	--	1	--	--	36	15. 0
	Granulocytic	--	--	--	--	--	--	--	--	--	--	--	--	None		--	--	--	--	--	--	--	--	--	--	--	--	----	----
	Reticulum-cell sarcoma	--	--	--	--	--	--	--	--	--	--	2	1	1	--	1	--	1	--	--	--	2	--	--	--	--	--	8	17. 1
	"Hodgkin's-like lesion"[1]	--	--	--	--	--	--	--	1	--	--	--	--	--	1	1	--	1	--	--	1	1	--	--	--	--	--	6	17. 6
$DWF_1 \times W$; $W \times DWF_1$	Lymphocytic	--	--	1	--	--	3	3	7	9	7	6	2	1	7	4	2	1	--	2	--	1	--	1	--	1	--	58	13. 2
	Granulocytic	--	--	--	--	1	--	--	--	1	--	--	--	--	1	1	--	--	--	--	--	--	--	--	--	--	--	4	12. 7
	Reticulum-cell sarcoma	--	--	--	--	--	--	--	1	--	--	--	3	--	--	--	--	1	1	1	--	--	--	1	--	--	--	8	17. 1
	"Hodgkin's-like lesion"	--	--	--	--	--	--	--	--	--	--	2	--	--	1	--	1	1	1	1	--	1	--	--	1	--	--	9	18. 8

[1] This refers to a pleomorphic lesion, described by Dr. Thelma B. Dunn, Laboratory of Pathology, as consisting of large, round, and fusiform cells which are occasionally multinucleate and large and small lymphocytes, plasma cells, and occasional neutrophillic and eosinophillic granulocytes. A complete morphologic description of lesions of this type will be published later by Dr. Dunn.

were found to be leukemic. The difference is statistically significant: 16.3 ± 6.9 percent; $P = .016$.

TABLE 3.—*Incidence of leukemia in DWF_1-W backcross mice classified as to genotype*

Genotype	Mice with leukemia		Nonleukemic mice	
	Number	Percent	Number	Percent
Sh-2 Wa-2 sh-2 wa-2	27	30. 7	61	69. 3
sh-2 wa-2 sh-2 wa-2	23	31. 5	50	68. 5
Sh-2 wa-2 sh-2 wa-2	5		20	
sh-2 Wa-2 sh-2 wa-2	14		28	
Sh-2 sh-2	32	28. 3	81	71. 7
sh-2 sh-2	37	32. 2	78	67. 8
Wa-2 wa-2	41	31. 5	89	68. 5
wa-2 wa-2	28	28. 6	70	71. 4
Ff	28	22. 8	95	77. 2
ff	41	39. 1	64	60. 9

DISCUSSION

Proof that genetic factors are influential in determining the susceptibility of mice to the development of spontaneous leukemia has been provided by the so-called "progeny test" (*7*). Breeding tests of the genotypes of 50 backcross males, obtained by crosses of high- (C58) and low-leukemic (Sto-Li) strains, revealed that many genotypes, resulting from segregation of genes, were to be found among these mice. Additional proof of the influence of genes is to be found in linkage data on susceptibility to leukemia and specific marked genes. The flexed-tail gene (*f*), although introduced into the cross from the low-leukemic strain WA, was found to be associated with an increased susceptibility to leukemia, among the backcross segregants studied. It would appear that the observed relationship between increased susceptibility to leukemia and the flexed-tail gene represents a condition of specific gene effect on susceptibility to leukemia, rather than a condition of linkage of susceptibility genes and the flexed-tail gene. If linkage were the explanation, normal (*Ff*), rather than flexed (*ff*), backcross segregants would be expected to show increased susceptibility to leukemia, since the normal allele (*F*) was introduced into the cross from the DBA strain, the high-leukemic strain. The situation is analogous to the effect of the yellow (A^y) gene in carcinoma of the breast (*10*) and lung adenoma (*13*, *14*) and to the dilution (*d*) gene in leukemia (*7*).

Two of the marked genes introduced into the cross reported here [flexed-tail (*f*) that increased susceptibility to leukemia, and shaker-2 (*sh-2*) that was ineffective in influencing susceptibility to leukemia] decreased susceptibility to development of lung adenoma (*14*) when introduced into another cross. These observations indicate that specific genes are related to the induction of specific neoplasms. Several workers in human genetics, notably Jacobson (*22*), assume, on the other hand, that a common genotype favors the induction of tumors in general.

It appears quite probable that leukemia in the mouse is a genetic entity. The genes which determine susceptibility to leukemia may not be specific as regards the morphologic type of the disease. In various crosses, F_1, F_2, and backcrosses to the low-leukemic strain, where the number of susceptibility genes is reduced, it is possible that other variables have an influence not only on the incidence of the disease (*7*) but in the induction of a specific cell type. Hogreffe (*4*) described the morphologic variations of the leukemia arising in various hybrid generations involving a cross with strain Ak mice, in which the predominant cell type is lymphocytic. The work of Videbaek (*23*) in familial cases of human leukemia also suggests that leukemia is a genetic entity.

The differences in incidence of leukemia in reciprocal F_1 hybrid mice support the observation of MacDowell (*7*, *21*) and the indications of others (*9*, *4*) of a specific maternal influence in leukemia. An analysis of the mechanism responsible for these differences is not possible with the present material.

SUMMARY

Reciprocal crosses were made between high-leukemic strain DBA and low-leukemic strain WA mice.

The incidence of induced leukemia was significantly higher in F_1 hybrids obtained from matings with the high-leukemic female.

Flexed-tail (*ff*) segregants, obtained in the backcross to the low-leukemic strain, were more susceptible to induced leukemia than were their normal sibs (F*f*). There was found no established relationship between susceptibility to leukemia and two other segregating genes, shaker-2 (*sh-2*) and waved-2 (*wa-2*).

The available evidence favors the assumption that the flexed-tail gene, *per se*, which is responsible for other symptoms in the mouse including a transitory siderocytic anemia, directly influences susceptibility to leukemia.

Considerably greater variation in mean age at death from leukemia, and in morphologic types of leukemia, appeared in the F_1 and backcross mice.

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TRANSFER OF THE MAMMARY TUMOR MILK AGENT FROM IMPLANT TO HOST [1]

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The literature concerning the mouse mammary tumor milk agent has been well reviewed (*1*). The usual mode of transmission is *via* the mother's milk, but laboratory transmission has been accomplished by the injection of any of a variety of "infected" materials. The experiments reported in this paper were designed to study some aspects of the transmission of the agent from mouse to mouse by the inoculation of agent-containing tissues. The principal points investigated were: (1) the use, as inoculum, of tumor tissue that would grow in the host and require later excision; (2) the interval, after inoculation, before the appearance of the milk agent in the tissues of the host; (3) the influence of splenectomy upon transfer of the agent; and (4) the relative resistance of adult mice, as contrasted with sucklings, to the injection of the agent.

All of the mice used in these experiments were born and raised in this laboratory. All were maintained under uniform environmental conditions, approximately 8 to a cage, in plastic cages with stainless steel tops. They were fed Purina laboratory chow and water *ad libitum*. The diagnoses of the mammary tumors were, in almost all cases, confirmed by study of histologic sections.

EXPERIMENT 1

This experiment concerns the use, as inoculum, of tumor tissue that would grow in the host and require later excision.

Andervont reported that if suckling (I × C3H) F_1 hybrid mice were inoculated intracutaneously with a mammary adenocarcinoma that would grow in the host, and that if the growing implant were later surgically excised, the inoculated mice would be found, for the most part, to have remained free of the effects of the agent. He obtained only 1 tumor in a series of 23 animals (*2*). Similar findings have been reported by Hummel (*3*). These results are not exactly in accord with previous work relating to the transplantation of milk agent-bearing tumors or tissues into suckling mice of susceptible strains. Previous experience has shown that a high percentage of susceptible suckling mice can be expected to develop tumors if they have been inoculated with any of a variety of agent-

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[2] National Institutes of Health, Public Health Service, Federal Security Agency.

containing materials (*1*). However, in such previous work the "infected" inoculum has not been placed intracutaneously, nor has it been excised after a relatively short period of residence in the susceptible animals. The findings reported by Andervont could be the result of any one or more of the following factors: (1) the intracutaneous site used; (2) the small dosage of tissue inoculated; (3) the short interval before excision; (4) the use as inoculum of mammary-tumor tissue that would grow in the test animals.

The experiment reported here was designed to ascertain primarily whether the growth of a subcutaneously implanted tumor would produce "infection" in the host.

Female mice of strain C (BALB/c), 3 to 22 days of age, were given mammary-tumor tissue from strain C_+ [BALB/c carrying the agent (*4*)] by inoculation into the subcutaneous tissues of the back with a 15-gauge trocar. Care was taken to use apparently healthy tissue. The pieces of tumor used were of a size that fitted snugly into the barrel of the trocar. Usually, several mice received inoculations from 1 tumor. It was thought to be advantageous to use a variety of agent-containing tumors of spontaneous origin that had not been previously transplanted, in order to avoid results dependent upon 1 tumor of perhaps unusual potency. The 16 spontaneous mammary tumors used arose in strain C_+ mice from 6 to 14 months of age.

The tumor implants in the experimental mice were allowed to grow until it was felt that further growth would cause ulceration or prohibit the possibility of successful surgical resection. They were excised under nembutal-alcohol anesthesia. In 9 cases the tumors ulcerated before operation. Some of the tumors recurred and were re-excised 2 to 4 weeks after the initial excision. The tumor implants remained in the hosts for periods of 19 to 90 days. Approximately one-half of the tumor-inoculated mice were splenectomized at the same time that their tumor implants were being excised. Throughout Experiment 1, all animals, at the conclusion of the experimental procedures, were mated and cast 1 litter.

The 56 mice inoculated with tumor, splenectomized as well as non-splenectomized, constitute series A. The results are summarized in table 1. Of the 56 mice, 35 reached tumor-age (considered as 7 months throughout this experiment) and 14 developed tumors. Thus, 40 percent of these animals were shown to have been "infected" by the temporary subcutaneous growth within them of agent-containing tumor tissue. Subsequent data will show that all, or almost all, of the mice of this series were actually harboring the agent even though only 40 percent developed tumors.

Some of the freshly excised tumor implants from the series A mice were inoculated by the previously described trocar-technique into suckling (I $\times$ C3Hb) F_1 hybrid females. These hybrids are sensitive test animals for the presence of milk agent and, due to genetic incompatibility, the excised tumor-implants from the strain C mice did not grow in them. These test animals comprise series B.

TABLE 1.—*Results in mice of Experiment 1*

	Number of mice in series	Number of mice of parent series represented in test series	Number of mice of parent series represented by tumors in test series	Number of tumors	Average tumor age (months)	Average age at death of nontumor-bearing mice (months)	Tumor percentage
Series A	35			14	14	16.5	40
Derived from series A:							
Series B	7	6	5	6	16	18	86
Series D	35	33	19	19	10	14	54
Series F	27	10	7	8	10	14	34
Series C	23			10	13	13.5	43
Derived from series C:							
Series E	17	17	6	6	9	13	35
Series G	7	2	1	5	11	15	71

The results of this series are seen in table 1. Unfortunately, only 7 mice reached tumor age, due to the occurrence of diarrhea of the newborn to which strain C3H mice and their hybrids are unusually susceptible (*5*). The results show conclusively, however, that the agent was present in the tumor-implants excised from the series A animals.

Series C was designed as a control for the efficacy, in general, of subcutaneous inoculation of tissue as a means of transmitting the agent. This series was composed, in large part, of litter mates of the mice that were inoculated with tumor tissue (series A). They were inoculated in identical fashion, but with spleen instead of tumor. The splenic tissue was obtained from the same animals which constituted tumor donors for series A. Whenever practical, each mouse of series C was paired with a corresponding mouse of series A throughout the experiment, and each was operated upon at the same time as its experimental sibling. No attempt was made to find or excise the splenic implant, but each mouse was relieved of its native spleen if that procedure had been followed with the companion mouse of the tumor-inoculated series.

The results of series C may be found in table 1. Forty-three percent of the mice developed tumors, as compared with 40 percent in series A. It is thus apparent that the results were quite similar, whether spleen was used as inoculum or tumor tissue that would grow in the host. The only difference between the two series was the age at death of the nontumor-bearing mice. It may be concluded from these experiments that mice can be infected with the milk agent in the same order of magnitude by inoculation with tumor tissue that will grow in the host as by inoculation of other "infected" tissues.

This conclusion is further confirmed by the results obtained by testing the tumor-producing capacity of the spleens that had been excised from approximately half the mice inoculated with tumor (series A) and half the mice inoculated with spleen (series C). Each spleen was inoculated into one or two strain C suckling females by the usual trocar-technique, to constitute series D and E, respectively. The results are shown in

table 1. Series D and E not only confirm the similarity of "infection" rates between series A and C, but also show that the agent was readily detectable in the spleens of the inoculated hosts (series A and C) within 3 weeks of the time of inoculation. The latter aspect has been analyzed in table 2.

TABLE 2.—*Experiments 1 and 2. Results of testing for the agent at intervals after its inoculation into suckling mice*

Experiment No.	Interval in days	Number of spleens tested	Number of test mice	Number of tumors	Average tumor age (months)	Average age death non-tumor-bearing mice (months)	Tumor percentage
2	7	4	7	1	14	17	14
1	19–23	30	30	13	9	14	43
1	24–36	20	22	10	10	14	45
2	37–45	6	8	8	10	----	100
2	74–90	4	6	2	16	17	33
1	365+	12	34	13	10	13	40
Total	----	76	107	47	10	14	44

It is of interest that the distribution of the tumors in the spleen-test mice (series D and E) had no relationship to the distribution of tumors in the mice from which the spleens had been obtained (series A and C), thus indicating that all, or almost all, of the series A and C mice were actually "infected," even though less than half of them developed mammary tumors. This assumption is further confirmed by the additional data of series F and G which follow.

Those animals that had originally been inoculated with either tumor or spleen (series A and C) and that had not been splenectomized were killed at 18 months of age, if they did not possess tumors, and their spleens tested. The spleens were tested by the usual trocar-technique using suckling strain C females as test mice. The test animals constitute series F and G, respectively, and the results obtained are shown in table 1. These data help to confirm the contention that mice of series A and C were, in fact, "infected" in the same order of magnitude. It is again evident that those mice of series A and C that did not develop tumors were, nevertheless, "infected." Andervont has recently emphasized the possibility that mice harboring the agent may not develop mammary tumors, and that one generation is inadequate to prove the absence of the milk agent (*6*).

The results of this experiment have shown under certain conditions the efficacy of using as agent-inoculum tumor tissue that would grow in the host and require later excision. They have shown that "infection" of the host takes place within 3 weeks and that the agent is detectable in the host spleens at that time. Furthermore, most of those hosts that did not develop tumors were shown to be "infected" by the presence of the agent in their spleens shortly after inoculation or at 18 months of age.

EXPERIMENT 2

This experiment concerns the interval of time following inoculation of the milk agent required for its appearance in the tissues of the host. An important characteristic of the agent is the long latent period between its inoculation into susceptible animals and tumor formation. Perhaps this latent period could be explained by a very slow rate of proliferation of the agent in the susceptible mouse. It was thought that an indication of the rate of increase of the agent might be gained by a study of the amount of time it would take for it to become detectable in a new host.

Twelve male strain C mice averaging about 3 weeks of age were used. They were divided into 3 groups of 4 each in such a way that litter mates were spread among the groups as evenly as possible. The mice were then inoculated with spleen obtained at random from either 1 or 2 strain C_+ females, which were themselves bearing spontaneous mammary tumors. The trocar technique used in Experiment 1 was again utilized. The first group of experimental mice was killed 1 week after the inoculation of spleen, the second group 6 weeks after, and the last group after 12 weeks. When the animals were killed, their spleens were tested for the presence of the agent by inoculation (same trocar technique) into suckling C or (I $\times$ C3Hb) F_1 hybrid females. The test mice in this experiment were allowed to produce 2 litters in rapid succession (force-breeding).

The results have been combined with similar data from Experiment 1 and are presented in table 2. These results support further the conclusion arrived at in Experiment 1 that, under the conditions employed, the agent is detectable in the new host within 3 weeks from the time it is introduced, and possibly earlier. This experiment was performed with males, and since the results parallel those obtained with females (Experiment 1), the findings suggest that males, without estrogen supplements, were as readily "infected" with the agent as were females.

EXPERIMENT 3

This experiment deals with the influence of splenectomy on transfer of the agent. Spleen is a convenient source of the agent (7) and was used as such in Experiments 1 and 2. It seemed advisable, therefore, to determine whether or not splenectomy itself would influence the "infection" rate.

Female strain C mice 1 month of age were splenectomized under nembutal-alcohol anesthesia and inoculated during the course of the operation with one fragment of a strain C3H mammary tumor by the trocar method. Four separate strain C3H spontaneous tumors were used, and these implants did not grow in the strain C hosts, due to their genetic incompatibility.

Littermate controls were subjected to mock operations in which their spleens were left intact. They were inoculated with agent in the same manner as the experimental mice and from the same tumor material.

In this experiment the mice were allowed to produce 2 litters in rapid

succession (force-breeding) before being put aside for observation of mammary-tumor development.

The results of Experiment 3 are summarized in table 3 where they have also been combined with data from Experiment 1. The low tumor

TABLE 3.—*Experiments 1 and 3. Effect of splenectomy on "infection" with the agent*

	Number of mice	Number of tumors	Average tumor age (months)	Average age at death nontumor-bearing mice (months)	Tumor percentage
Splenectomized 3–4 weeks after inoculation of agent (Experiment 1)	31	12	13	13	39
Splenectomized at time of inoculation of agent (Experiment 3)	28	6	13	19	21
Total for splenectomized	59	18	13	16	31
Nonsplenectomized (Experiment 1)	27	12	13	18	45
Nonsplenectomized (Experiment 3)	28	6	11	20	21
Total for nonsplenectomized	55	18	12	19	33

incidence in this experiment was probably due to the fact that the mice were not inoculated with agent until they were a month of age. From the combined results of Experiments 1 and 3, as seen in table 3, it is apparent that splenectomy, either at the time of, or at an average of 3 weeks after agent inoculation did not alter the "infection" rate.

EXPERIMENT 4

This experiment concerns the relative resistance of adult mice, as contrasted with suckling mice, to the injection of the agent. It seems to be well established that adult mice are, in general, less affected by inoculation of agent-containing materials (*2, 8–12*). It is not clear, however, whether the absence of mammary tumors is due to failure of the mice to become "infected" with the agent, or whether it is due to a lack of sensitivity to its action. An attempt was made in this experiment to compare the "infectivity" of the agent in adult and suckling mice, as distinguished from its possible tumor-producing action in such animals. A further purpose was to determine whether or not the relative resistance of adult mice could be reduced by partial suppression of reticulo-endothelial activity by the injection of trypan blue.

Strain C female mice averaging 7 months of age were divided into two series. Each of these mice had had 2 to 4 litters prior to its use in this experiment. In the trypan blue series (series A), 0.5 cc. of a 0.5 percent aqueous solution of trypan blue was injected intraperitoneally into 21 adult females. A total of 4 injections was given to each mouse at 2-day intervals (one injection every other day). Coincidentally, with the third injection of dye, the mice were inoculated subcutaneously with a fragment of mammary tumor and a fragment of spleen from a strain

C3H mouse. A 12-gauge trocar was used. Note that this is a variation in inoculation technique from that used in the previous experiments. The larger trocar and the two fragments were employed to compensate roughly for the fact that the adult animals used in this experiment were heavier than the sucklings inoculated in the previous work. Two strain C3H mice bearing spontaneous tumors were used as tissue donors.

Series B consisted of 10 animals treated identically to those in series A, except that no trypan blue was injected.

The mice of both series were killed and their spleens tested for the presence of the agent at intervals of 1 week, 2 weeks, 4 weeks, 8 weeks, and 11 to 12 months after injection of the "infected" tissues. Mice of the 11- to 12-month group bore one litter after the inoculations were completed.

When a mouse was killed its spleen was removed aseptically, and cut into pieces suitable for use in a 15-gauge trocar. Test animals were 1- to 3-weeks-old strain C or (I × C3H) F_1 hybrid females. Each test animal was inoculated subcutaneously with two or three pieces of spleen, each piece being derived from a different mouse. The test animals were bred, allowed to give birth to one litter and then observed for tumor-formation.

One of the mice of series A developed a tumor before its scheduled date of execution.

Table 4 shows the results in the test mice. Those derived from series A and B have been combined since there was no significant difference between them. Only two tumors appeared. These occurred in a group of three test animals, each of which had been inoculated with three fragments of spleen, one from each of three adults of series A. Thus, it must be presumed that at least one of the adults had been infected. The fact that all of the positive cases were derived from trypan-blue-injected mice was presumably due to chance.

TABLE 4.—*Experiment 4. Results of testing for the agent at intervals after its inoculation into adult mice*

Interval in days	Number of spleens tested	Number of test mice	Number of tumors	Average tumor age (months)	Average age nontumor-bearing mice (months)	Tumor percentage
7	4	7	0	----------	13	----------
14	5	6	0	----------	16	----------
28	7	7	0	----------	17	----------
56	8	6	[1] 2	9	19	33
335+	7	13	0	----------	9	----------
Total	31	39	2	9	13	5

[1] Both tumor-bearing mice had been inoculated with spleen fragments from the same three mice of series A (see text). These three mice of series A had been inoculated with agent-containing material from a different strain C3H donor than had been used for the one series A mouse which had itself developed a tumor. This is evidence that both of the strain C3H agent-donors used in this experiment were, in fact, carrying the agent.

When the results of this experiment (table 4) are compared with the data in table 2, it is apparent that the adult mice did not become "infected" to the same degree as did the sucklings. Not only did the adults fail to develop tumors, but they also apparently lacked the milk agent.

DISCUSSION

It is apparent from the data of Experiment 1 that there was no significant difference in "infection" rate between those mice inoculated subcutaneously with tumor, which was later excised, and those inoculated with a similar amount of spleen. In the former case (series A) in a total of 35 animals the tumor incidence was 40 percent. In the latter case (series C) the tumor incidence in 23 animals was 43 percent. In evaluating these data, the discrepancy in the average age at death of non-tumor-bearing mice between the two series (16. 5 months and 13.5 months, respectively) must be borne in mind. The similarity in results is striking, however, if the other series are also taken into account. Thus, in the mice inoculated with tumor (series A) and the mice inoculated with the spleens excised from them (series D and F), a total of 97 animals reached tumor age of which 41 or 42 percent developed tumors; while in the mice inoculated with spleen (series C) and the spleen-test mice derived from them (series E and G), of 47 mice that reached tumor age, 21 or 44.7 percent developed tumors.

Thus, the data are not sufficient to exclude the possibility of a real difference in "infection" rate between those mice inoculated with tumor tissue and those inoculated with spleen, but do suggest that if such a difference existed, it was of small magnitude. It seems certain that the results are quite different from those obtained by Andervont. However, his mice were not tested for the presence of the agent, and may actually have been "infected" even though they did not develop tumors.

The influence of the length of time that the tumor implants remained in their series A hosts on the incidence of tumors was determined. Those mice in which the tumor implants recurred after initial excision and required a second operation were compared with those in which such recurrence did not take place. In the former case, the tumors remain in the hosts an average of 42 days, while in the latter, the average stay was only 22 days. The difference in tumor incidence between these two groups was suggestive but not statistically significant. In those mice in which the implant recurred, 6 of 12 developed mammary tumors. Eight of 23 of the mice in which the implant did not recur developed tumors. It is probable that if the length of stay of the implants in their hosts had been markedly reduced (below the range used in this experiment), this would have become a significant factor. Since the interval between inoculation and excision was comparable in this experiment to that reported in the work of Andervont, it is apparent that the length of time of exposure to the tumor cells cannot account for the difference in results.

The experiment reported here differed from Andervont's in three essentials: the size of the tumor inoculum, the site inoculated, and the strain of mice used. The last point serves only to accentuate the difference in results rather than to account for it, since the strain C mice used in the present work are regarded, in this laboratory, as somewhat more resistant to the agent than the hybrids used by Andervont.

These experiments do not clarify the significance of the site and the

dose as factors in the difference between the present results and those of Andervont. Perhaps both are important. However, there are some facts that bear indirectly upon this question. It is well known from numerous studies on bacterial infection that the skin of most animals is more resistant to a foreign agent than are most other sites. In an immune host this differential effect is amplified. The skin of the immune rabbit has the property of localizing the invading organism or toxin and keeping it from spreading throughout the animal (*13*).

Similarly, the skin is more resistant to tumor transplantation. Inoculation into this site often allows the host to build an immunity to the foreign cells that would have been impossible under other conditions (*14*, *15*). It is, therefore, possible that the skin of the mice in Andervont's experiment localized the agent until the agent-bearing tumor was excised.

It has not been definitely established whether the milk agent multiplies in tumor tissue, although the agent has been shown to persist through many transplant generations in mice presumably free of "infection" (*2*, *16*). Andervont (*3*), however, reported contrary results and Barnum, Ball and Bittner (*17*) stated that although spontaneous tumor is a good source of the agent, transplanted tumor tissue is relatively poor. If the agent does multiply or increase at a rapid rate in growing tumor tissue, the dosage of tumor inoculated in Andervont's experiment would not be a factor in the results, providing, of course, that the agent escaped from the local site.

The dosage of tumor inoculated could be a factor in another way. Perhaps it is necessary to destroy some of the tumor cells in order to allow the agent to escape to the host. In that event, a large inoculum of tumor would be of importance since it is probable that, even in the case of autologous tumor transplantation, only the more peripheral cells of a sizable transplant survive. It was with this possibility in mind that nine of the mice in the tumor-inoculated series (series A) were permitted to carry their implants until ulceration (when presumably many tumor cells would be killed). However, the generally positive results made analysis of this factor impossible from the available data.

The results of Experiment 1 suggest that Andervont's findings were not dependent upon any hypothetical protection against "infection" with the agent which might have been conferred by the presence of an actively growing mammary tumor-implant, *per se*, nor were they dependent on the length of time *per se* that the animals were exposed to the agent-bearing material. His findings must have depended upon the dosage and/or the site of inoculation. Further work is necessary to determine the relative importance of these two factors.

It should be mentioned that Hummel (*2*) has reported a similar type of experiment in which tumor tissue was inserted subcutaneously into the ears of mice, and the ears were subsequently amputated. Only 2 of 34 mice developed mammary tumors. Inoculation in the thin tissues of the ear was probably comparable to Andervont's intracutaneous injection. The paper does not report the length of time the grafts remained in the

hosts. Evaluation is further complicated by the fact that the tumors used were previously transplanted. Furthermore, almost one-half the implants regressed spontaneously.

The results of Experiments 1 and 2 established the fact that the mouse mammary tumor milk agent was detectable in the spleens of inoculated suckling mice within 3 weeks after inoculation of the agent (in the dosage employed) and probably earlier (table 2). This is an interesting result in view of the suggested hypothesis that the agent increases slowly throughout the life of the animal.

In Experiment 1, of the original mice that had been inoculated with tumor or spleen (series A and C), 24 of 58, or 41 percent, developed tumors. Of those mice that had been inoculated with splenic tissue excised at an early age from the animals of series A and C (series D and E), 25 of 52, or 48 percent developed tumors. Of the mice of series F and G that had been inoculated with spleen which had been excised at 18 months from the series A and C mice, 14 of 34, or 41 percent developed tumors. Thus, it is apparent that for practical purposes (tumor production) the dosage of agent obtained from the spleens and tumors of the original strain C_+ tumor-donors was equivalent to that obtained from series A and C mice, regardless of the length of the time interval from inoculation of series A and C mice to the time the agent was obtained from them. At first glance, this result seems to indicate that the agent increased to a constant maximal level within 3 to 10 weeks of its inoculation, *i. e.*, that it was capable of rapid increase. Another interpretation, however, is that in each case the dosage obtained, though probably small when obtained from 3 to 10-week-old series A and C mice, was above the minimum required to produce a maximal tumor response. In other words, according to this interpretation, a relatively small dosage, could, under the conditions of this experiment, lead to a maximal tumor incidence. Differences in dosage, therefore, would be masked and would probably be significant, or detectable by the techniques used in this work, only at the extremes of the dosage scale. Such an interpretation is compatible with a slow rate of increase of the agent.

It also follows from the data, that all, or almost all, of the mice implanted with agent-bearing tumor or spleen (series A and C) were "infected" by the inoculation, even though the resulting tumor incidence was less than 50 percent. This conclusion necessarily follows from the fact that the tumor incidence resulting from the testing of their spleens (series D, E, F and G) was identical with their own tumor incidence. Since they had been inoculated directly from mice of strain C_+, all of which must be presumed to have been "infected," and since the resulting tumor incidence was between 40 and 50 percent, it follows that the series A and C mice, in turn, must have been almost 100 percent infected in order to have produced a similar tumor incidence when their own spleens constituted the donor tissue. Substantiation of this conclusion was seen in the tumor distribution data of spleen test series D, E, F and G in which

most of the nontumor-bearing mice were actually tested and demonstrated to have been carrying the agent.

Experiments 1 and 3 (table 3) show that splenectomy, under the conditions employed, did not have an observable influence on the incidence or course of "infection" with the agent, regardless of whether the operation was performed at the time of, or after, inoculation. Thus, of 59 mice which were inoculated with tumor or spleen and were splenectomized, 18 or 31 percent developed tumors. Of 55 comparable control animals, 18 or 33 percent developed tumors.

Experiment 4 suggests that the failure of adult mice of an otherwise susceptible strain to develop tumors after inoculation with the agent is probably due primarily to the fact that these mice seldom become "infected" to the same degree as do sucklings (*cf.* tables 2 and 4). This result should not be interpreted as indicating complete absence of the milk agent in the adult mice. A more sensitive testing technique might well have given positive results. All that was actually demonstrated was a relative absence.

It could be argued that Experiments 1 and 2 did not constitute good controls for Experiment 4, inasmuch as the inoculum used was different: in the former case, strain C spleen, and in the latter, strain C3H tumor tissue and spleen. However, strain C3H tumor tissue has been generally accepted as a potent source of the agent (*10*) and was shown to be such in Experiment 3 of this paper.

Inasmuch as two separate donors were used, the possibility of the results having been due to a poor source of agent seems very remote. It should be noted also that the inoculum used in Experiment 4 was larger (1 piece of spleen and 1 piece of tumor in a 12-gauge trocar) than that used in Experiments 1 and 2 (1 piece of spleen in a 15-gauge trocar). This was done to compensate, roughly, for the difference in size between adult and suckling mice. Test animals in Experiment 4 received a larger dose of spleen than did the test animals in Experiments 1 and 2. Thus, a modest attempt to favor "infection" of mice in Experiment 4 was not successful. Dmochowski (*18*) has shown, however, that very large doses of agent-containing spleen can produce tumors in adult mice. Whether the tumors in the mice of his experiment resulted from a multiplication of agent in the hosts (active infection) or from the action of the actual amount of agent injected is not known.

Vital staining with trypan blue did not influence the relative resistance of adult mice to infection with the agent. It can well be argued that a different dosage schedule might have produced different results. The literature on this subject has recently been assembled by Borges (*19*).

SUMMARY AND CONCLUSIONS

The transfer of the mammary tumor milk agent from a tissue implant to the host has been studied.

It was found that, even if the implant were composed of tumor tissue that would grow in the suckling host and require later excision, the transfer

of the agent took place readily. This was demonstrated by the tumor incidence in the implanted strain C hosts, as well as by the agent in their spleens. This result was contrasted with those of Andervont and of Hummel who had reported a poor transmission under somewhat similar conditions. It is concluded that Andervont's observations were dependent upon dosage and/or site of inoculation, rather than upon the nature of the inoculum or the length of time *per se* that the implant remained in the host.

It was also found that the milk agent was detectable in the spleens of the strain C suckling hosts within 3 weeks (and probably sooner) after the subcutaneous inoculation of milk agent-bearing tissue. There was no observed sex difference in this respect.

After its inoculation, the agent appeared rapidly in the spleens of suckling mice, whereas it was seldom found in spleens of inoculated adults. Furthermore, administration of trypan blue did not increase the susceptibility of adult hosts to the agent.

Splenectomy did not influence the transmission of the agent from implant to host.

Many animals that did not develop tumors were shown to be "infected," demonstrating that one nontumor-bearing generation is insufficient to prove that a mouse is free of mammary tumor milk agent.

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INTERRELATION OF SPECIFIC GENES, BODY WEIGHT, AND DEVELOPMENT OF TUMORS IN MICE [1]

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An association between susceptibility to induced pulmonary tumors in mice and the flexed-tail gene, and also the linked genes shaker-2 and waved-2, has been demonstrated (*1*). In the backcross groups tested, the flexed-tailed (*f/f*) segregants were less susceptible to the development of pulmonary tumors following intravenous injection of 1: 2: 5: 6-dibenzanthracene than were the normal-tailed (*F/f*) segregants, and the shaker-2 waved-2 (*sh-2 wa-2/sh-2 wa-2*) segregants were less susceptible than were those bearing the normal alleles (*Sh-2 Wa-2/sh-2 wa-2*). In both cases the normal alleles came from the susceptible strain, while the recessive gene came from resistant strains. Whether the relationships were true linkage, *i. e.*, tumor susceptibility genes located on the chromosomes bearing the genes tested, or the reduction in susceptibility was due to the tested genes *per se* was not ascertained.

Following the demonstration of these relationships, it was desirable to extend the project to spontaneous pulmonary tumors to ascertain whether or not these particular or tested genes were likewise related to the spontaneous tumors. Earlier it had been shown that the lethal yellow (A^y) gene increased susceptibility to both induced and spontaneous pulmonary tumors (*2, 3*) and hairless (*hr*) was associated with a decrease in susceptibility to both (*4, 5*). Since there was a suggestion of a positive correlation between the association of the gene with susceptibility and its effect upon body weight in that lethal yellow increased body weight and hairless reduced it, the present tests with flexed-tail, shaker-2 and waved-2 also included studies on body weight. The mice comprising these tests were permitted to live as long as possible and, thus, data on tumors other than pulmonary tumors were obtained. Data presented herein demonstrate that the flexed-tail, shaker-2 and waved-2 genes do have the same relationship to spontaneous pulmonary tumors as they have to the induced tumors and also uphold the positive correlation between the relationship of the gene to body weight and to susceptibility to pulmonary tumors. No relationship to other types of tumors was revealed.

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EXPERIMENTAL PROCEDURES

Strain A mice were used as representatives of a strain highly susceptible to pulmonary tumors. This strain has been used extensively in this laboratory in studies on the inheritance of this type of tumor. It has been shown that approximately 90 percent of the mice of this strain have spontaneous pulmonary tumors at 18 months of age (*6*), and following the intravenous injection of 0.5 mg. 1:2:5:6-dibenzanthracene pulmonary tumors occur in 100 percent of the injected animals at 6 weeks. At 4 months, the lungs of each mouse have approximately 75 tumor nodules (*7*). At the time these experiments were started, strain A had been inbred 64 brother × sister generations, and thus, could be considered homozygous for as nearly all gene pairs as possible.

Strain A mice were outcrossed to mice of strain WA. This strain, WA, was obtained from Dr. G. D. Snell, who had fixed the three recessive genes, flexed-tail, shaker-2, and waved-2 in the strain. The strain had not been inbred, although it was homozygous for these three genes. Although the exact incidence of pulmonary tumors in the strain was not ascertained, it was known to be relatively low.

The F_1 hybrids produced by outcrossing strain A to strain WA were backcrossed to the parent strain WA. In this WA-backcross a 50:50 ratio would be expected, between flexed-tail (*f*/*f*) and non-flexed (*F*/*f*), shaker (*sh-2*/*sh-2*) and non-shaker (*Sh-2*/*sh-2*), and between waved (*wa-2*/*wa-2*) and non-waved (*Wa-2*/*wa-2*). The shaker-2 and waved-2 genes are linked, while the flexed-tail gene segregates independently. With the crossover groups there would thus be eight genotypic groups, all of which could be identified phenotypically. As shown by Hunt, Mixter, and Permar (*8*), however, normal *f*/*f* overlaps occur, but these can be identified by the fact that all *f*/*f* mice are anemic at birth and, therefore, can be detected easily at that time.

At birth, the strain WA-backcross mice were examined, and all anemic mice were identified by clipping the right toe of the right posterior foot. Of 33 second and third litters, all were individually identified by clipping the toes and were weighed at birth. At 4 weeks of age all were weaned and further classified as to genotype with respect to *f*, *sh-2*, and *wa-2*. Those classified as *f*/*f*, because of the anemia at birth were further classified as to whether they were phenotypically flexed or normal *f*/*f* overlaps, *i. e.*, genotypically flexed but with normal tails.

The mice were segregated as to sex and set aside in plastic cages with eight mice to each cage. They were given an unlimited supply of tap water and of pelleted Derwood laboratory feed. They were weighed at the time of weaning and at weekly intervals thereafter to 28 weeks, after which they were weighed at 4-week intervals.

An autopsy was done on each animal when it was found dead or moribund with a tumor, or from some other cause. The lungs were examined for tumors with the aid of the dissecting microscope. All tumors were fixed in Tellyesniczky's fluid (70 percent ethyl alcohol, 20 parts; formalin,

2 parts; glacial acetic acid, 1 part). They were mounted in paraffin, sectioned, and stained with hematoxylin and eosin.[3]

In order to ascertain the effect of the waved-2 gene without the shaker-2 gene, strain A mice were also outcrossed to a waved strain obtained from Dr. S. C. Reed. This strain, although not highly inbred, was homozygous for the waved-2 gene, and was homozygous for the normal allele of the linked-gene shaker-2, *i. e.*, *Sh-2 wa-2/Sh-2 wa-2*. The strain was relatively low in susceptibility to pulmonary tumors as compared with strain A, but its response to the intravenous injection of 0.5 mg. 1:2:5:6-dibenzanthracene indicated that its degree of susceptibility was not as low as some of the highly resistant strains, such as strain C57 L. At 6 months following intravenous injection of the carcinogen, 80 percent of a group of the waved-strain mice had pulmonary tumors and an average of 2 tumor nodules in the lungs of each mouse.

The F_1 hybrids were backcrossed to the parent waved strain to obtain the backcross generation for the linkage test. In this waved-backcross, one would expect a 50:50 ratio between waved (*wa-2/wa-2*) and non-waved (*Wa-2/wa-2*), although all would be normal with respect to shaker (*Sh-2/Sh-2*). These backcross mice were treated in a manner similar to that of the former experiment, except that in this case the animals were kept in wooden cages, were fed Purina laboratory chow, and all were killed and examined at 18 months of age.

RESULTS

The occurrence of tumors in the various genotypic groups is given in table 1. The tumor-age listed was the age at autopsy at which time the tumor was found, except in the case of the mammary-gland tumors in which the tumor-age was the age at which the tumor was first observed. An excess of mice can be noted in the group heterozygous for the three dominant genes. This probably was the result of a reduction in viability caused by the recessive genes when homozygous.

In order to ascertain whether or not there was a relationship between either of the three genes tested and susceptibility to any of the various types of tumors, the incidence of each tumor in all mice homozygous for the recessive gene tested was compared with that in all mice heterozygous for the dominant allele. Such comparison was also made between the sexes. These tumor incidences are listed in table 2, along with the probabilities that the differences in incidence between the genotypic and sex groups were due to chance.

There is evidence that the flexed-tail gene is associated with susceptibility to spontaneous pulmonary tumors just as it was formerly shown to be associated with induced pulmonary tumors. When the incidence of all *f/f* mice is compared with all *F/f* mice the probability value is between the 2- and 3-percent levels. This value is reduced, however, to between 1 and 2 percent when only those phenotypically flexed are compared with normal *F/f*. In view of the fact that flexed-tail was shown to be associated with

[3] The authors gratefully acknowledge aid from Dr. Thelma Dunn, Pathologist, National Cancer Institute, in the diagnosis of the tumors.

TABLE 1.—*Occurrence of tumors in the backcross mice*

Genotype	Sex	Total mice		Pulmonary tumors		Mammary tumors		Hepatoma		Tumors of blood-forming organs		Others		
		Number	Average age (*Months*)	Number	Average age (*Months*)	Number	Average age (*Months*)	Number	Average age (*Months*)	Number	Average age (*Months*)	Number	Type	Age (*Months*)
WA backcross:														
F/f Sh-2 Wa-2/sh-2 wa-2	♂	44	18.8	20	19.6	0	------	4	18.8	2	23.5	1	Hemangio-endothelioma.	23
	♀	50	17.1	23	19.1	30	14.9	0	------	7	20.6	1	Granulosal cell tumor of ovary.	23
F/f sh-2 wa-2/sh-2 wa-2	♂	34	15.6	11	15.4	0	------	1	16.0	0	------			
	♀	32	16.1	7	17.7	17	12.5	1	22.0	3	17.3	1	Fibrosarcoma of cervix.	19
F/f Sh-2 wa-2/sh-2 wa-2	♂	8	12.8	2	16.0	0	------	0	------	0	------	1	Hemangio-endothelioma.	21
	♀	16	14.1	9	16.2	12	13.1	0	------	0	------			
F/f sh-2 Wa-2/sh-2 wa-2	♂	13	21.3	4	20.5	0	------	1	23.0	0	------			
	♀	23	19.6	7	23.1	7	9.9	0	------	4	21.5	1	Sarcoma of pancreas.	24
												1	Mast cell tumor of lung.	26
f/f Sh-2 Wa-2/sh-2 wa-2 (phenotypically flexed-tailed)	♂	12	18.6	5	18.4	0	------	1	20.0	1	20.0			
	♀	20	18.2	8	19.5	11	17.5	1	22.0	2	21.0	1	Adenoma of Harderian gland.	17
												1	Hemangio-endothelioma.	20
												1	Subcutaneous sarcoma.	22
f/f Sh-2 Wa-2/sh-2 wa-2 (normal *f/f* overlaps)	♂	21	17.6	9	18.4	0	------	2	16.5	0	------			
	♀	23	18.3	5	18.8	13	16.8	0	------	2	19.0	1	Adenoma of islet of Langerhans.	20
f/f sh-2 wa-2/sh-2 wa-2 (phenotypically flexed-tailed)	♂	9	13.3	0	------	0	------	0	------	0	------			
	♀	11	13.6	1	18.0	5	12.0	0	------	0	------			
f/f sh-2 wa-2/sh-2 wa-2 (normal *f/f* overlaps)	♂	14	15.2	5	16.2	0	------	1	19.0	0	------	1	Hemangio-endothelioma.	19
	♀	8	16.6	2	17.5	3	12.0	1	22.0	1	24.0			

f/f Sh-2 wa-2/sh-2 wa-2 (phenotypically flexed-tailed)	♂	4	15.8	2	16.5	0	------	0	------	0	------			
	♀	10	14.2	0	------	6	11.3	0	------	0	------			
f/f Sh-2 wa-2/sh-2 wa-2 (normal *f/f* overlaps)	♂	4	15.2	1	21.0	0	------	0	------	1	21.0			
	♀	0	------	0	------	0	------	0	------	0	------			
f/f sh-2 Wa-2/sh-2 wa-2 (phenotypically flexed-tailed)	♂	5	18.4	0	------	0	------	1	15.0	1	21.0			
	♀	5	18.2	1	21.0	3	17.3	0	------	1	18.0			
f/f sh-2 Wa-2/sh-2 wa-2 (normal *f/f* overlaps)	♂	4	20.7	1	22.0	0	------	1	21.0	1	21.0			
	♀	5	17.4	1	25.0	4	15.5	0	------	1	19.0	1	------do------	19
Waved backcross:														
Wa-2/wa-2	♂♀	22	18.0	9	18.0	0	------	2	18.0	0	------			
		23	18.0	6	18.0	0	------	0	------	2	18.0			
wa-2/wa-2	♂♀	17	18.0	10	18.0	0	------	0	------	1	18.0			
	♀	14	18.0	3	18.0	0	------	1	18.0	1	18.0			

TABLE 2.—*Comparison of incidence of tumors in strain WA backcross segregants*

Type of tumor	Segregants				X^2	P
	Genotype	Incidence of tumors	Genotype	Incidence of tumors		
		(*percent*)		(*percent*)		
Pulmonary tumor	*F/f*	37. 7	*f/f*	26. 5	5. 22	0. 02<*P*<0. 03
	F/f	37. 7	*f/f*[1]	22. 4	5. 96	.01<*P*<.02
	F/f	37. 7	*f/f*[2]	30. 1	1. 37	.2 <*P*<.3
Mammary tumor	*F/f* (♀ ♀)	54. 5	*f/f* (♀ ♀)	52. 2	.075	.7 <*P*<.8
Hepatoma	*F/f*	3. 18	*f/f*	5. 16	.93	.3 <*P*<.5
Pulmonary tumor	*Sh-2/sh-2*	39. 6	*sh-2/sh-2*	24. 5	9. 47	*P*<.01
Mammary tumor	*Sh-2/sh-2* (♀ ♀)	60. 5	*sh-2/sh-2* (♀ ♀)	46. 4	3. 35	.05<*P*<.1
Hepatoma	*Sh-2/sh-2*	3. 77	*sh-2/sh-2*	4. 29	1. 16	.2 <*P*<.3
Pulmonary tumor	*Wa-2/wa-2*	37. 3	*wa-2/wa-2*	26. 7	4. 63	.03<*P*<.04
Mammary tumor	*Wa-2/wa-2* (♀ ♀)	54. 0	*wa-2/wa-2* (♀ ♀)	55. 8	.07	.7 <*P*<.8
Hepatoma	*Wa-2/wa-2*	4. 89	*wa-2/wa-2*	2. 67	1. 16	.2 <*P*<.3
Pulmonary tumor	♂ ♂	34. 9	♀ ♀	31. 5	.47	.3 <*P*<.5
Hepatoma	♂ ♂	5. 8	♀ ♀	1. 5	5. 23	.02<*P*<.03

[1] Phenotypically flexed-tail.

[2] Normal overlaps.

induced pulmonary tumors, together with all the parallelisms demonstrated between the genetics of induced and spontaneous pulmonary tumors, it is justifiable to consider these differences with their respective probability values as highly significant. It is to be noted, however, that when *F/f* mice are compared with the *f/f* normal overlaps a significant difference is not found. The probability value is between 20 and 30 percent.

Results of these tests also show that the shaker-2 gene is associated with susceptibility to pulmonary tumors as it was shown to be associated with induced pulmonary tumors. The shaker-2 (*sh-2/sh-2*) segregants had a significantly lower incidence than that of the normal (*Sh-2/sh-2*) segregants. The difference between the incidence of pulmonary tumors of the segregants homozygous for waved-2 (*wa-2/wa-2*) that is linked with shaker-2 and the normal (*Wa-2/wa-2*) segregants is not so highly significant. It should be noted also in table 1, that when the waved animals (*wa-2/wa-2*) of the second experiment were compared with normal (*Wa-2/wa-2*) in the absence of the shaker-2 gene no difference at all was indicated.

There was no evidence of a sex difference in incidence of pulmonary tumors. The probability value in table 2 is between 30 and 50 percent. This is in line with published data on this tumor (*5*, *9*).

Since females of strain A were used in the outcross to strain WA and the F_1 females were backcrossed to strain WA males, the mammary tumor agent would be expected to have been transmitted from the strain A females through the F_1 females to the backcross animals. Fifty-five percent of these backcross females developed mammary-gland tumors. Since these backcross females were kept as virgins, however, and since strain A virgin females have a low incidence of mammary tumors, these backcross females must have inherited genes from their strain WA fathers suitable for this relatively high incidence in virgins. Differences in incidence in table 2, however, do not give evidence of any association between mammary tumors and the three genes, flexed-tail, shaker-2 or waved-2.

Hepatomas were observed in the backcross mice of both experiments, but the incidence was not high and did not offer any evidence of association with any of the three genes tested. A sex difference in incidence of hepatomas was apparent, however, in the strain WA backcross group, the males having a significantly higher incidence than that of the females. There were not enough hepatomas in the waved-strain backcross for significant comparison. The sex difference observed here is in line with that observed elsewhere (*5*, *10*).

Tumors listed as tumors of the blood-forming organs in table 1 included reticulum-cell sarcomas, lymphocytic leukemia, and one case that was probably granulocytic leukemia. These were placed under one heading because of the difficulty in making a precise separation between these different neoplasms of blood-forming organs when sections only are available. Intermediate forms between the well-differentiated types exist. Relatively few were well-differentiated lymphocytic leukemia, and the major portion were reticulum-cell sarcoma, applying this term to neoplasms originating from the reticulum cells of the lymphoid tissue. These

neoplasms fall in a group described by Gorer (*11*) as histiocytomas, and by Lewis (*12*) and Furth and Furth (*13*) as monocytomas. The occurrence of these reticulum-cell sarcomas in the old backcross mice is in line with the general observation that this type of tumor generally occurs in older mice, whereas lymphocytic leukemia usually occurs in younger animals. The data on these tumors of the blood-forming organs, however, were not extensive enough for analysis of linkage.

Other tumors occurring in the strain-WA-backcross group are listed in table 1. While some of these, such as the adenoma of the Harderian gland and the adenoma of the Islet of Langerhans, were interesting because they occur infrequently in mice, none offered data sufficient for linkage analyses.

ANALYSIS OF WEIGHTS

The weight data of the strain-WA-backcross mice were analyzed for the purpose of making six independent comparisons of weight curves for mice with contrasting characteristics, *e. g.*, males *vs.* females, mice with mammary tumors *vs.* mice without mammary tumors, mice with pulmonary tumors *vs.* mice without pulmonary tumors, mice with normal tails *F/f vs.* mice with flexed tails *f/f*, non-shaker mice *Sh-2/sh-2 vs.* shaker mice *sh-2/sh-2*, mice with normal coats *Wa-2/wa-2 vs.* mice with waved coats *wa-2/wa-2*.

For this purpose it is not sufficient to sort the animals into two contrasting groups and to compare the weight curves for each group. Thus, if we compare all males with all females, the latter group will include animals with mammary-gland tumors while the former will not. Differences between the two weight curves might then be ascribable to the effect of sex or to the effect of mammary-gland tumors. For this particular case, it would be sufficient to eliminate animals with mammary-gland tumors from the comparison, in which case apparent sex differences could no longer be ascribed to the effect of mammary-gland tumors. Even then, however, if any of the other characteristics occurred more frequently in one sex than another, an apparent sex difference might actually be due to the effects of this other characteristic.

What is required for this analysis then is a method of making each of the 6 independent comparisons in such a fashion as to systematically eliminate the effect of the other five.[4] This can always be done by restricting the comparison to animals that are the same with respect to the other 5 characteristics not being compared. Thus, there are 20 males and 10 females with lung tumors with the genotype *F/f Sh-2 Wa-2/sh-2 wa-2*, and without mammary-gland tumors (table 3). Differences in weight curves between these 2 groups do not reflect the effects of any of the other 5 characteristics. But this is only 1 of the combinations of the 5 characteristics for which males and females can be compared. Another is the 23 males and 9 females without pulmonary tumors with the genotype *F/f Sh-2 Wa-2/sh-2 wa-2* and without mammary-gland tumors. In all, there are 32 different

[4] The same method is also required for the analysis of differences in tumor incidence of the preceding section. The more elaborate method does not change any of the conclusions reached and is consequently not presented.

Age at death (weeks)	Pulmonary tumor						
	F/f				*f/f*		
	Sh-2/sh-2		*sh-2/sh-2*		*Sh-2/sh-2*		*sh-2/*
	Wa-2/wa-2	*wa-2/wa-2*	*Wa-2/wa-2*	*wa-2/wa-2*	*Wa-2/wa-2*	*wa-2/wa-2*	*Wa-2/wa-2*
Over 72	9	2	0	0	6	0	0
66–71	1	0	0	0	1	0	0
60–65	0	0	0	1	0	0	0
54–59	2	1	0	0	0	0	0
48–53	1	1	0	1	1	0	0
42–47	0	1	0	0	0	0	0
36–41	0	1	0	0	0	0	0
30–35	0	0	0	0	1	0	0

Age at death (*weeks*)

Over 72
66–71
60–65
54–59
48–53
42–47
36–41
30–35

TABLE 3.—*Number of animals in each characteristi*

FEMALES

Mammary gland tumor											
No pulmonary tumor											
F/f						*f/f*					
sh-2/sh-2		*Sh-2/sh-2*		*sh-2/sh-2*		*Sh-2/sh-2*		*sh-2/sh-2*		*Sh-2/sh-2*	
Wa-2/wa-2	*wa-2/wa-2*	*Wa-2/wa-2*	*wa-2/wa-2*	*Wa-2/wa-2*	*wa-2/wa-2*	*Wa-2/wa-2*	*wa-2/wa-2*	*Wa-2/wa-2*	*wa-2/wa-2*	*Wa-2/wa-2*	*wa-2*
0	0	1	0	0	2	7	0	3	0	9	
0	0	2	0	0	1	2	0	1	1	0	
0	0	3	1	0	1	0	0	1	1	1	
0	0	4	0	1	1	3	0	0	2	0	
0	0	3	4	0	3	2	3	0	2	0	
0	0	1	0	2	2	1	2	1	0	0	
0	0	2	0	2	1	0	1	1	0	0	
0	0	1	0	1	1	0	0	0	1	0	

MALES

	Pulmonary tumor					
	F/f				*f/f*	
	Sh-2/sh-2		*sh-2/sh-2*		*Sh-2/sh-2*	
	Wa-2/wa-2	*wa-2/wa-2*	*Wa-2/wa-2*	*wa-2/wa-2*	*Wa-2/wa-2*	*wa-2/wa-2*
----------	15	0	4	4	11	2
----------	2	1	0	1	1	1
----------	1	0	0	2	1	0
----------	1	0	0	1	1	0
----------	1	0	0	2	0	0
----------	0	1	0	1	0	0
----------	0	0	0	0	0	0
----------	0	0	0	0	0	0

TABLE 3.—*Number of animals in each characteristic subclass*

FEMALES

No pulmonary tumor						Pulmonary tumor					
		f/f				*F/f*				*f/f*	
sh-2/sh-2		*Sh-2/sh-2*		*sh-2/sh-2*		*Sh-2/sh-2*		*sh-2/sh-2*		*Sh-2/sh-2*	
Wa-2/wa-2	*wa-2/wa-2*	*Wa-2/wa-2*	*wa-2/wa-2*	*Wa-2/wa-2*	*wa-2/wa-2*	*Wa-2/wa-2*	*wa-2/wa-2*	*Wa-2/wa-2*	*wa-2/wa-2*	*Wa-2/wa-2*	*wa-2/wa-2*
0	2	7	0	3	0	9	2	7	4	4	0
0	1	2	0	1	1	0	1	0	0	0	0
0	1	0	0	1	1	1	0	0	1	0	0
1	1	3	0	0	2	0	0	0	0	0	0
0	3	2	3	0	2	0	0	0	0	0	0
2	2	1	2	1	0	0	0	0	0	0	0
2	1	0	1	1	0	0	0	0	0	0	0
1	1	0	0	0	1	0	0	0	0	0	0

MALES

Pulmonary tumor							
F/f			*f/f*				
sh-2/sh-2			*Sh-2/sh-2*		*sh-2/sh-2*		*Sh-2/sh-2*
wa-2/wa-2	*Wa-2/wa-2*	*wa-2/wa-2*	*Wa-2/wa-2*	*wa-2/wa-2*	*Wa-2/wa-2*	*wa-2/wa-2*	*Wa-2/wa-2*
0	4	4	11	2	1	3	16
1	0	1	1	1	0	0	2
0	0	2	1	0	0	0	2
0	0	1	1	0	0	1	0
0	0	2	0	0	0	0	1
1	0	1	0	0	0	1	0
0	0	0	0	0	0	0	1
0	0	0	0	0	0	0	1

No mammary gland tumor										
			No pulmonary tumor							
f/f			*F/f*				*f/f*			
	sh-2/sh-2		*Sh-2/sh-2*		*sh-2/sh-2*		*Sh-2/sh-2*		*sh-2/sh-2*	
2	*Wa-2/wa-2*	*wa-2/wa-2*	*Wa-2/wa-2*	*wa-2/wa-2*	*Wa-2/wa-2*	*wa-2/wa-2*	*Wa-2/wa-2*	*wa-2/wa-2*	*Wa-2/wa-2*	*wa-2/wa-2*
0	2	2	7	0	9	8	11	3	1	5
0	0	0	1	0	0	1	2	0	0	1
0	0	0	0	0	0	1	1	0	0	2
0	0	1	1	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	1	0	0
0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0

No pulmonary tumor						
F/f			*f/f*			
sh-2	*sh-2/sh-2*		*Sh-2/sh-2*		*sh-2/sh-2*	
wa-2/wa-2	*Wa-2/wa-2*	*wa-2/wa-2*	*Wa-2/wa-2*	*wa-2/wa-2*	*Wa-2/wa-2*	*wa-2/wa-2*
1	9	8	10	1	5	3
0	0	4	1	0	1	1
0	0	4	4	1	2	3
0	0	1	2	1	0	4
3	0	2	0	2	0	3
0	0	2	1	0	0	3
0	0	1	1	0	0	0
1	0	0	0	0	0	1

possible combinations of the 5 characteristics on which males and females might be compared (2 for pulmonary tumors $\times$ 2 for *f* $\times$ 2 for *sh-2* $\times$ 2 for *wa-2* $\times$ 2 for mammary-gland tumors), although obviously not all these classes need have both males and females in them. In fact, only 14 of these possible 32 have both males and females. The 16 classes with mammary-gland tumors contain no males, and in addition, the following 2 classes contain no females: (1) without mammary-gland tumors, with pulmonary tumors, *f/f Sh-2 wa-2/sh-2 wa-2*, and (2) without mammary-gland tumor, without lung tumor, *F/f Sh-2 wa-2/sh-2 wa-2*. In practice, therefore, we can find 14 comparisons of male and female weight curves for which all 5 other characteristics are held constant. In general, the effect of sex need not be the same for all 14 classes. There may be an interaction between the effect of sex and 1 or more of the other characteristics. We are not concerned here with the interactions, but only with the main effect of sex. The main effect of sex is the average difference between weight curves through all 14 classes.

There is one other complication. The age at death varies considerably and, of course, influences weight at ages prior to death. Furthermore, age at death is related to some of the characteristics under study. The proportion of males surviving beyond the 72d week, for example, is much smaller than the proportion of females. In consequence, age at death is another variable that it becomes necessary to control, even though its effect on weight is not of concern. Table 3 shows the number of animals in each of the homogeneous subclasses obtained by classifying animals by age at death and each of the six variables under study.

To obtain an estimate of the main effect of any one of the six characteristics, it is then merely necessary to find subclasses which are comparable with respect to the other five. Thus, in table 4, all comparable subclasses required for the male-female comparison are shown. (Subclasses not shown either have no males, no females, or none.) In the last two columns we show average animal weight at 30 weeks for each subclass separately for males and females. It is apparent that, at least at this age, the females tend to weigh less than the males. To estimate the average difference, *i. e.*, the main effect of sex, one might compute the average difference for each subclass and average these averages. This would give equal weight to subclasses with differing numbers which would, of course, be undesirable. We have consequently used a weighting factor, given in column 3, which increases with increasing numbers of animals.[5] The average body weight computed using the factors is 42.8 gm. for males and 38.0 gm. for females, and the average difference is 4.8 gm. Proceeding in this fashion for each age we obtain the results given in table 5.[6]

[5] The variance of the difference between two means is $\sigma_1^2/n_1+\sigma_2^2/n_2$. If we have a series of differences, each based on differing numbers of animals, an appropriate way to combine them is to weight each difference by the reciprocal of its sampling variance. On the assumption that the σ's are equal, this gives as weighting factors $1/(1/n_1+1/n_2)$, which is the factor given.

[6] When we come to age 54 the subclass consisting of animals that died at age 48 to 53 drops out. From this age on, averages are computed on a chain basis.

TABLE 4.—*Comparable subclasses for male—female comparison*

Age at death, in weeks	Characteristics	Number animals		Weighting factor (1) × (2) / (1) + (2)	Average animal weight at 30 weeks	
		Male (1)	Female (2)	(3)	Male	Female
					Grams	*Grams*
Over 72	Pulmonary tumor:					
	F/f Sh-2/sh-2 Wa-2/wa-2	15	9	5.6	49.3	42.9
	F/f sh-2/sh-2 Wa-2/wa-2	4	7	2.5	39.5	35.0
	F/f sh-2/sh-2 wa-2/wa-2	4	4	2.0	42.0	37.8
	f/f Sh-2/sh-2 Wa-2/wa-2	11	4	2.9	44.7	44.5
	f/f sh-2/sh-2 Wa-2/wa-2	1	2	.7	36.0	26.0
	f/f sh-2/sh-2 wa-2/wa-2	3	2	1.2	41.7	33.0
	No pulmonary tumor:					
	F/f Sh-2/sh-2 Wa-2/wa-2	16	7	4.9	45.9	42.6
	F/f sh-2/sh-2 wa-2/wa-2	9	9	4.5	39.3	35.7
	F/f sh-2/sh-2 wa-2/wa-2	8	8	4.0	40.5	35.8
	f/f Sh-2/sh-2 Wa-2/wa-2	10	11	5.2	45.9	41.3
	f/f Sh-2/sh-2 wa-2/wa-2	1	3	.8	37.0	37.3
	f/f sh-2/sh-2 Wa-2/wa-2	5	1	.8	36.6	28.0
	f/f sh-2/sh-2 wa-2/wa-2	3	5	1.9	40.3	26.4
66–71	Pulmonary tumor:					
	F/f Sh-2/sh-2 wa-2/wa-2	1	1	.5	33.0	29.0
	No pulmonary tumor:					
	F/f Sh-2/sh-2 Wa-2/wa-2	2	1	.7	52.0	38.0
	F/f sh-2/sh-2 wa-2/wa-2	4	1	.8	39.5	25.0
	f/f Sh-2/sh-2 Wa-2/wa-2	1	2	.7	50.0	42.5
	f/f sh-2/sh-2 wa-2/wa-2	1	1	.5	47.0	25.0
60–65	Pulmonary tumor:					
	F/f Sh-2/sh-2 Wa-2/wa-2	1	1	.5	42.0	47.0
	F/f sh-2/sh-2 wa-2/wa-2	2	1	.7	29.0	42.0
	No pulmonary tumor:					
	F/f sh-2/sh-2 wa-2/wa-2	4	1	.8	41.2	50.0
	f/f Sh-2/sh-2 Wa-2/wa-2	4	1	.8	44.5	43.0
	f/f sh-2/sh-2 wa-2/wa-2	3	2	1.2	38.7	32.5
50–59	Pulmonary tumor:					
	f/f sh-2/sh-2 wa-2/wa-2	1	1	.5	32.0	33.0
48–53	No pulmonary tumor:					
	f/f Sh-2/sh-2 wa-2/wa-2	2	1	.7	38.0	24.0

TABLE 5.—*Average weight, males and females*

Age (*weeks*)	Sum of weighting factors	Males	Females
		Grams	*Grams*
6	45.4	23.8	21.0
12	45.4	31.8	26.4
18	45.4	36.8	31.6
24	45.4	40.4	34.7
30	45.4	42.8	38.0
36	45.4	45.2	40.8
42	45.4	45.5	40.7
48	45.4	45.9	41.8
54	44.7	45.6	42.5
60	44.2	45.5	42.5
66	40.2	43.9	42.0
72	37.0	41.0	41.4

By regrouping the subclasses and repeating the process, averages may now be computed for any other pair of contrasting characteristics, as shown in tables 6 to 10. All differences in tables 3 to 8 eliminate the effect of all characteristics mentioned except the one being considered.

TABLE 6.—*Average weight, animals with and without pulmonary tumors*

Age (*weeks*)	Sum of weighting factors	Pulmonary tumors	No pulmonary tumors
		Grams	*Grams*
6	57. 9	22. 7	22. 8
12	57. 9	29. 7	29. 5
18	57. 9	34. 9	34. 7
24	57. 9	38. 8	38. 1
30	57. 9	41. 6	40. 5
36	57. 9	44. 6	42. 7
42	57. 9	44. 9	42. 9
48	56. 4	45. 7	43. 9
54	51. 8	46. 1	44. 1
60	48. 5	45. 9	43. 7
66	44. 7	45. 4	42. 7
72	41. 0	43. 3	41. 1

TABLE 7.—*Average weight, normal (Sh-2/sh-2) and shaker (sh-2/sh-2)*

Age (*weeks*)	Sum of weighting factors	*Sh-2/sh-2*	*sh-2/sh-2*
		Grams	*Grams*
6	47. 3	22. 6	21. 9
12	47. 3	30. 0	27. 3
18	47. 3	35. 6	31. 5
24	47. 3	39. 5	34. 7
30	46. 3	42. 3	36. 3
36	45. 8	45. 2	38. 8
42	44. 8	45. 7	38. 9
48	43. 1	46. 6	39. 8
54	37. 3	46. 9	40. 4
60	35. 7	46. 9	40. 6
66	33. 1	46. 0	39. 8
72	31. 4	43. 1	38. 6

TABLE 8.—*Average weight, normal (F/f) and flexed-tail (f/f)*

Age (*weeks*)	Sum of weighting factors	*F/f*	*f/f*
		Grams	*Grams*
6	65. 5	23. 4	21. 6
12	65. 5	30. 2	28. 0
18	65. 5	35. 6	32. 8
24	65. 5	38. 9	36. 8
30	65. 5	41. 6	39. 4
36	65. 0	44. 2	41. 9
42	63. 8	44. 4	41. 8
48	60. 9	45. 3	42. 6
54	53. 9	46. 1	42. 3
60	49. 7	45. 3	42. 0
66	45. 0	44. 0	41. 6
72	39. 1	42. 0	39. 4

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TABLE 9.—*Average weight, normal (Wa-2/wa-2) and waved (wa-2/wa-2)*

Age (*weeks*)	Sum of weighting factors	*Wa-2/wa-2*	*wa-2/wa-2*
		Grams	*Grams*
6	42. 5	22. 6	21. 2
12	42. 5	28. 8	28. 0
18	42. 5	33. 6	32. 9
24	42. 5	37. 7	36. 0
30	41. 5	40. 0	38. 1
36	40. 5	42. 5	40. 6
42	39. 8	42. 5	40. 7
48	38. 1	43. 5	40. 6
54	33. 9	44. 4	40. 5
60	32. 0	44. 2	41. 4
66	28. 7	43. 5	40. 0
72	26. 5	42. 6	37. 8

TABLE 10.—*Average weight, females with and without mammary-gland tumors*

Age (*weeks*)	Sum of weighting factors	Mammary-gland tumor	No mammary-gland tumor
		Grams	*Grams*
6	22. 0	21. 7	20. 6
12	22. 0	27. 6	26. 3
18	22. 0	32. 2	32. 1
24	22. 0	36. 8	35. 8
30	21. 5	38. 9	39. 5
36	21. 5	40. 7	42. 6
42	21. 5	41. 2	42. 4
48	21. 5	41. 5	43. 5
54	20. 7	42. 3	44. 5
60	19. 9	41. 6	44. 1
66	18. 2	41. 1	43. 7
72	15. 5	41. 0	42. 6

SAMPLING ERROR

While it is possible to compute the standard error of each average in tables 5 to 10, this would represent a considerable amount of work. The following approximate procedure seems adequate. If the standard deviation of each subclass were constant, the standard error of any difference in tables 5 to 10 would be that standard deviation divided by the square root of the sum of the weighting factors. Spot computations of the standard deviations for various subclasses show that it varies from 10 to 20 percent of the mean of the class. If the standard deviation at 20 percent of the mean is taken, a conservative estimate may be had. On this basis, the difference between male and female, between *Sh-2/sh-2* and *sh-2/sh-2*, and between *F/f* and *f/f* are significant at the 1 percent level. The difference between mammary-gland tumors and no mammary-gland tumors is definitely not significant even at the 5 percent level.[7] The difference between pulmonary tumors and no pulmonary tumors and *Wa-2/wa-2* and *wa-2/wa-2* are significant at the 5 percent level, but not at the 1 percent level.

[7] The fact that animals with mamary-gland tumors weigh consistently less than those without them at all ages from 30 weeks on does not provide additional evidence, since the differences at different ages are not independent of each other and do not, therefore, constitute additional items of evidence.

Birth Weight

The birth-weight data for the animals of the experiment that were weighed at birth was tabulated and combined in accordance with the above system of weights. When the eventual duration of life of the animal was disregarded, the weights, in grams, at birth were as presented in table 11, where also is listed the approximate standard errors of the differences for each comparison, determined with an estimate of the standard deviation as about 20 percent of the mean.

The difference between males and females, animals with and without pulmonary tumors, females with and without mammary-gland tumors, and *Sh-2/sh-2* and *sh-2/sh-2* are not significant. The difference between *Wa-2/wa-2* and *wa-2/wa-2* is significant at the 5-percent level, and the difference between *F/f* and *f/f* is significant at the 1-percent level. Birth-weight differences for *Wa-2/wa-2* and *wa-2/wa-2*, and *F/f* and *f/f* are in the same direction as those later found when comparing animals after growth.

DISCUSSION

Through the demonstration of an association between specific genes of the mouse and susceptibility to pulmonary tumors the question is raised as to whether this association represents true linkage, *i. e.*, a gene for susceptibility located on the same chromosome as the specific gene tested, or that the effect on susceptibility is due to the specific gene *per se*. In the case of the lethal-yellow gene there is evidence that the effect is due to the gene itself. The difference associated with the gene was observed in F_1 hybrids that differed only in respect to this specific gene with the possible exception of closely linked genes. Furthermore, in another cross, agouti (an allele of lethal yellow) had no effect. There is also evidence that the reduction in susceptibility associated with the hairless gene may be an additional effect of this gene. In earlier tests (*14*) there was no evidence that piebald which is linked with hairless was related to susceptibility. Thus, the effect on susceptibility must have been due to the hairless gene *per se*, or else the susceptibility gene was located so far from the piebald locus that linkage to piebald could not be detected.

Data presented herein indicate also the action of the genes tested rather than true linkage. There was a significant reduction in susceptibility in animals that were phenotypically, as well as genotypically, flexed-tail (*f/f*), but not in the animals genotypically flexed (*f/f*) but phenotypically normal, *i. e.*, the normal overlaps. Thus, true linkage with susceptibility would be ruled out, and a direct association between the flexed-tail gene and susceptibility would be indicated. It is, furthermore, indicated that susceptibility was more directly related to the flexed-tail manifestation of the gene than to the anemia manifestation. The fact that all *f/f* mice are anemic at birth, whereas only a part of them are flexed-tailed, indicates that the anemia manifestation is rather closely related to the primary action of the gene, whereas the flexed-tail manifestation is more distantly related, thus permitting modifying factors to interfere with the chain of reactions in certain *f/f* animals before the flexed-tail trait is

TABLE 11.—*Comparison of birth weights of mice*

Characteristic	Average weight	Characteristic	Average weight	Difference	Sum of weighing factors	Standard error
	Grams		*Grams*	*Grams*		
Males	1. 615	Females	1. 536	0. 079	23. 2	0. 0651
With pulmonary tumors	1. 609	Without pulmonary tumors	1. 605	. 004	29. 8	. 0589
Females with mammary tumors	1. 609	Females without mammary tumors	1. 535	. 074	15. 3	. 0804
Sh-2/sh-2	1. 584	*sh-2/sh-2*	1. 598	−. 014	25. 4	. 0631
Wa-2/wa-2	1. 645	*wa-2/wa-2*	1. 517	. 128	25. 3	. 0629
F/f	1. 685	*f/f*	1. 462	. 223	33. 6	. 0543

formed. It would appear that these modifying factors, whatever they may be, also interfere with the reduction in susceptibility, suggesting that the control of susceptibility also is distantly related to the primary gene action, and that the connecting chain of reactions contains at least certain links of the chain of reactions leading to the abnormal tail-structure.

It is also apparent that the association between shaker-2 and susceptibility was due to the shaker-2 gene. When waved-2 that is on the same chromosome with the shaker-2 locus was tested in the absence of the recessive shaker-2 gene, no evidence of an association between waved-2 and susceptibility was shown. When waved-2 was tested in crosses also involving shaker-2 an association between waved-2 and susceptibility was indicated, although it was not as significant as the association with shaker-2.

Analyses of the growth curves of the animals of the present tests adds further evidence to that indicating a relationship between the effect of specific genes on susceptibility to pulmonary tumors and their effect on normal growth as indicated by body weight. Earlier (*2*, *3*) it has been shown that the lethal-yellow gene, that has long been known to increase body size and obesity, markedly increases susceptibility to pulmonary tumors. In other tests (*4*, *5*), it was shown that the hairless gene that decreased body weight decreased susceptibility to pulmonary tumors, whereas the pink-eye gene that had no influence on body weight likewise did not alter susceptibility. In the present analysis, flexed-tail and shaker-2, both of which decreased susceptibility, also decreased body weight. Waved-2, however, also reduced body weight but not as markedly as did shaker-2 and flexed-tail.

If certain genes were influencing susceptibility to pulmonary tumor-formation through their influence on normal growth, one would expect that of this genetically heterogeneous group of backcross mice those that weighed more would have a greater probability of developing pulmonary tumors. When the growth curves of those that developed pulmonary tumors were compared with the curves of the ones that did not, it was found that the tumor-mice weighed more, the difference being significant at the 5-percent level. However, there was no difference in weight between females that developed mammary-gland tumors and those that did not. There was a sex difference in weight but this was apparently due to factors unrelated to pulmonary tumor development, for there was no sex difference in susceptibility to pulmonary tumors.

It is evident that birth weight had no effect upon susceptibility to tumors. There was not a significant difference between the birth weights of those that developed pulmonary tumors and those that did not, nor was there any difference between birth weights of females that developed mammary-gland tumors and those that did not. Furthermore, the shaker-2 gene that was associated with a reduced susceptibility to pulmonary tumors had no effect upon birth weight. The flexed-tail gene that was also associated with a reduced susceptibility, however, did decrease birth weight. The fact that flexed-tail reduced birth weight,

whereas shaker-2 did not, can probably be explained by the fact that at birth there is no visible effect of the shaker-2 gene, whereas all flexed-tail animals are anemic at birth.

SUMMARY

There was an association between susceptibility to spontaneous pulmonary tumors of the mouse and the flexed-tail gene which apparently is attributable to the action of the gene *per se*, rather than to true linkage. Susceptibility was more closely associated with the flexed-tail manifestation of the gene than with the anemia manifestation.

The shaker-2 gene was likewise associated with susceptibility to spontaneous pulmonary tumors apparently also due to the action of the gene *per se*. The association between the waved-2 gene and susceptibility was apparently, therefore, the result of the linkage between waved-2 and shaker-2.

There was no evidence of any association between any of these three genes and susceptibility to mammary-gland tumors or to hepatomas.

There was not a significant sex difference in the incidence of pulmonary tumors, but the males had a higher incidence of hepatomas than did the females.

Analysis of weight curves revealed that both the flexed-tail and shaker-2 genes significantly reduced body weight. The waved-2 gene also reduced body weight, although not as much as did flexed-tail and shaker-2.

Mice that developed pulmonary tumors weighed more than those that did not, but there was no difference between the weight of females that developed mammary-gland tumors and those that did not. Males weighed more than females.

Flexed-tail significantly reduced birth weight, and so did waved-2, although not as much as did flexed-tail. Shaker-2 had no effect on birth weight, and sex had no effect on birth weight. There was no difference in birth weight between mice that developed pulmonary tumors and those that did not, and no difference in birth weight between females that developed mammary-gland tumors and those that did not.

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